Pyroptosis/Caspase-1 Assay

Far Red; Catalog #9158

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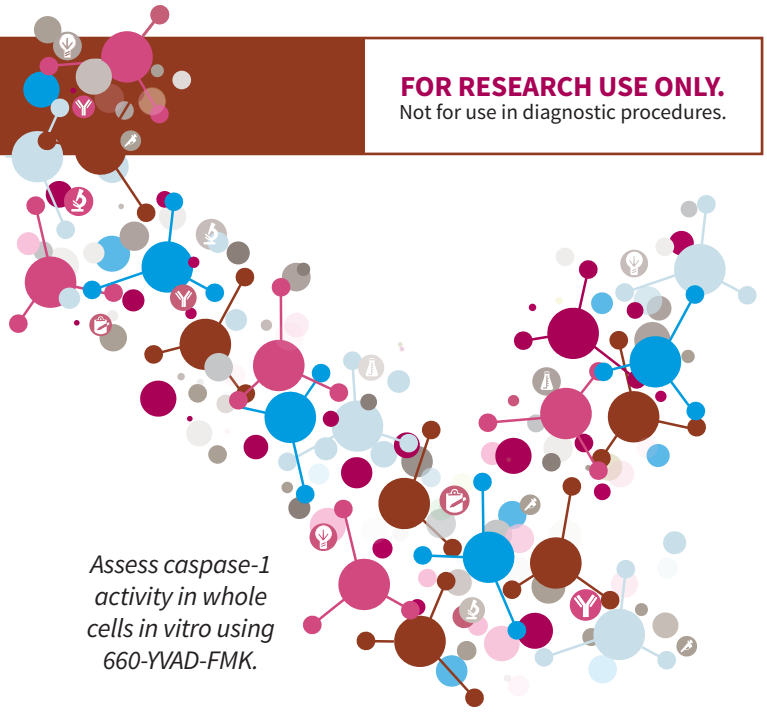
Not for use in diagnostic procedures.



Assess caspase-1 activity in whole cells in vitro using 660-YVAD-FMK.

1. INTRODUCTION

Exposure of inflammatory effector cells like monocytes and macrophages to pathogen-associated molecular patterns (PAMPS), such as viral or bacterial DNA or RNA and bacterial cell wall components like LPS, will typically trigger conformational changes in NACHT leucine-rich repeat protein family (NLRP) proteins[1,2]. Of the inflammasomes, the NLRP3 inflammasome is the most studied. Activation of the NLRP3 inflammasome follows exposure to PAMPS (first signal) and ATP (second signal) (Figure 1). This leads to oligomerization and assembly of a high molecular weight (~700 kDa) multimeric inflammasome complex, which leads to the conversion of pro-caspase-1 into the catalytically active form. Inflammatory caspases, such as caspase-1, or interleukin-converting enzyme, play a central role in innate immunity by recognizing foreign danger signals and initiating a two-fold response. First, caspase-1, proteolytically converts the proforms of the two important pro-inflammatory cytokines, interleukin 1ß (IL-1ß) and interleukin 18 (IL-18), into their active forms, which are secreted. Second, caspase-1 or caspase-11 triggers a form of lytic, programmed cell death known as pyroptosis[3,4].

Pyroptosis is a highly inflammatory form of programmed cell death that occurs most frequently upon infection with intracellular pathogens and is likely to form part of the antimicrobial immune response. This pathway is distinct from apoptotic cell death in that it results in plasma-membrane rupture and the release of pro-inflammatory cytokines; infected cells eventually swell, burst, and die. This, in turn, attracts other immune cells to fight the infection, leading to inflammation of the tissue, and, in a functional response, rapid clearance of bacterial or viral infections.

ICT's Pyroptosis/Caspase-1 Assay Kit utilizes our popular FLICA® technology to detect caspase-1 activation. FLICA probes are cell permeant noncytotoxic **F**luorescent **L**abeled **I**nhibitors of **CA**spases that covalently bind with active caspase enzymes[5,6]. The kit contains the caspase-1 inhibitor reagent 660-YVAD-FMK, which has the preferred binding sequence for caspase-1, Tyr-Val-Ala-Asp (YVAD)[7]. This preferred caspase-1 binding sequence is labeled with a far red fluorescent dye and linked to a fluoromethyl ketone (FMK) reactive entity. Caspase-1 will not cleave the FLICA inhibitor probe; instead, it forms an irreversible covalent bond with the FMK group on the reagent and becomes inhibited from further enzymatic activity.

To use FLICA, add it directly to the cell culture medium, incubate, and wash. FLICA is cell-permeant and will efficiently diffuse in and out of all cells. If there is an active caspase-1 enzyme inside the cell, it will covalently bind with 660-YVAD-FMK and retain the far red fluorescent signal within the cell. Unbound FLICA will diffuse out of the cell during the subsequent wash steps. Therefore, positive cells will retain a higher concentration of FLICA and fluoresce brighter than negative cells. There is no interference from pro-caspases or inactive forms of the enzymes. After labeling with FLICA, cells can be counter-stained with other reagents and fixed or frozen.

Cells labeled with 660-YVAD-FMK can be counter-stained with reagents such as the red live/dead stains Propidium Iodide (catalog #638) and 7-AAD (catalog #6163). Nuclear morphology may be concurrently observed using Hoechst 33342 (included in the kit), a blue DNA-binding dye. Cells can be viewed through a fluorescence microscope (Figures 2 and 4) or flow cytometer (Figures 3 and 5). FLICA 660 optimally excites at 660 nm and has a peak emission at 685-690 nm.

Nigericin, a potent microbial toxin derived from *Streptomyces hygroscopicus*, acts as a potassium ionophore, inducing a net decrease in intracellular levels of potassium which is crucial for oligomermization of the NLRP3 inflammasome and activation of caspase-1. Nigericin requires signaling through pannexin-1 to induce caspase-1 activation and IL-1ß processing and release. Nigericin is included in this kit as a positive control. It has been shown to generate a robust caspase-1 activation response in various cell lines, including Jurkat and THP-1 cells (Figures 3-5). FLICA® is for research use only. Not for use in diagnostic procedures.

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2. KIT CONTENTS

- 1 vial of 660-YVAD-FMK caspase-1 inhibitor reagent, #6323
- 1 bottle of 10X Cellular Wash Buffer (15 mL), #6164
- 1 bottle of Fixative (6 mL), #636
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 vial of Nigericin, 0.5 µmoles, #6698

3. STORAGE

Store the unopened Nigericin at -20°C until the expiration date. Store the remaining unopened kit components at 2-8°C until the expiration date. Once reconstituted with DMSO, use FLICA immediately, or aliquot and store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time. Reconstituted Nigericin is stable for up to 1 year at -20°C. Avoid repeated freeze/thaws; aliquot and store frozen.

4. SAFETY DATA SHEETS (SDS)

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

5. RECOMMENDED MATERIALS

- DMSO, 50 µL to reconstitute FLICA
- DiH_20, 135 mL to dilute 10X Cellular 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 treated with the experimental conditions ready to be labeled
- Reagents to induce caspase-1 activity to create a positive control; Nigericin (included in the kit) is one option
- Hemocytometer
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1 per sample)

6. DETECTION EQUIPMENT

The assay can be analyzed with a:

- Fluorescence microscope
- Flow cytometer

Use filter pairings that best approximate these settings:

- FLICA 660 excites at 660 nm and has a peak emission at 685-690 nm.
- Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 13).

7. EXPERIMENTAL PREPARATION

Staining caspase-1 positive cells with 660-YVAD-FMK can be completed within a few hours. However, since it is used to label living cells, adequate time needs to be allotted for the acquisition of functionally appropriate cells and expansion of a cell line known to produce caspase-1. The optimal cell concentrations and sample volumes will vary based on the experimental conditions and method of analysis.

In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment or caspase-1 induction process, which may take from several hours to multiple days, depending on the methods that are used. 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

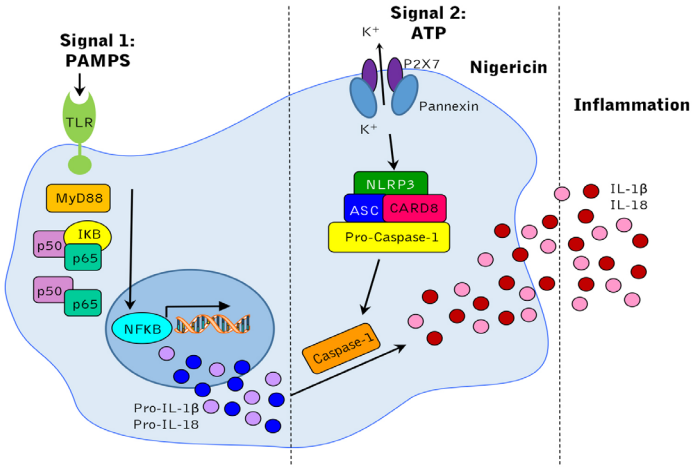
As 660-YVAD-FMK preferentially detects the presence of catalytically active forms of caspase-1, plan the experiment so that FLICA will be diluted and administered at the time when caspase-1 is expected to be activated in the cells. If this is not known, FLICA can be added so that it is present throughout the induction treatment period for up to 24 hours. Protect samples from light during that time.

The recommended volume of 30-60X FLICA is 5-10 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 experiment or caspase-1 induction protocol. Carefully monitor the density of adherent cell monolayers to avoid excess levels of confluency. Depending upon the cell line and type, cultivated cells which have reached a confluent monolayer may become spontaneously

FIGURE 1: ACTIVATION OF THE NLRP3 INFLAMMASOME

Oligomerization of the NLRP3 inflammasome is triggered by two signals. The first signal begins with the recognition of PAMPS (pathogen associated molecular patterns) by Toll-like receptors (TLRs), such as TLR4, which, through an interaction with the adapter protein MyD88, triggers activation of the transcription factor NF-κB. Once activated, NF-κB is translocated to the nucleus, where it leads to the synthesis of the inactive pro-inflammatory cytokine pro-IL-1ß. Another potent pro-inflammatory cytokine precursor, pro-IL-18, is constitutively expressed; however, its expression is increased after cellular activation. The second signal is triggered by an ionic perturbation in the cell, such as an efflux of K^+, caused by the ATP-dependent activation of the purinergic P2X receptor, which subsequently results in the assembly of the NLRP3 inflammasome, caspase-1 activation, and IL-1ß and IL-18 secretion.



apoptotic and trigger multicaspase activity. Cell density should not exceed 10^6 cells/mL in suspension cultures. Cells cultivated in excess of this concentration may naturally begin to 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.

8. 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 culture environment
c. Positive control: cells induced for caspase-1 activity using a known caspase-1 induction protocol

A common pool of cells should be used to generate the positive and negative control populations for FLICA, as they should contain similar quantities of cells. For example, when labeling with FLICA, make 4 populations:

1&2. Unlabeled: induced and non-induced

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

Note: If dual staining samples for flow cytometry analysis, additional single and dual-stained controls will need to be prepared to assist with compensation.

9. CASPASE-1 INDUCTION

The optimal 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 ng/mL Phorbol myristate acetate (PMA) in cell culture media for 48 hours (until cells become adherent), followed by exposure to 10-100 ng/mL Lipopolysaccharide (LPS) for ≥ 1 hour. In THP-1 monocytes, caspase-1 activity can be induced with 100 ng/mL LPS and 5 mM Adenosine triphosphate (ATP) for 24 hours, or 1 µg/mL LPS for 3 hours. Alternatively, Nigericin (included in the kit) can be used to generate a positive control; see next section for details.

10. PREPARATION OF NIGERICIN

Nigericin, a potent microbial toxin derived from *Streptomyces hygroscopicus,* acts as a potassium ionophore, inducing a net decrease in intracellular levels of potassium which is crucial for oligomerization of the NLRP3 inflammasome and activation of caspase-1 (Figure 1). Nigericin requires signaling through pannexin-1 to induce caspase-1 activation and IL-1ß processing and release. Nigericin is included in this kit as a positive control. It has been shown to generate a robust caspase-1 activation response in various cells lines, including Jurkat and THP-1 cells.

Nigericin is supplied lyophilized at 0.5 µmoles per vial. It may be slightly visible as an iridescent sheen or white powder inside the vial. Protect from light and use gloves when handling.

1. Reconstitute Nigericin with 100 µL DMSO to form the 5 mM stock concentrate. Once reconstituted, it may be aliquoted and stored at ≤-20°C for 1 year protected from light and thawed no more than twice during that time.
2. Immediately prior to addition to the samples and controls, dilute 5 mM Nigericin stock 1:10 in diH_2O to form a 500 µM working solution for use in treating samples. For example, dilute 1:10 by adding 20 µL stock concentrate to 180 µL diH_2O.
3. Use Nigericin at 1-20 µM to induce NLRP3 inflammasome and caspase-1 activation in cells. For example, to use at 10 µM, dilute 500 µM working solution 1:50 in samples; e.g., spike 294 µL cell suspension/overlay medium with 6 µL of 500 µM working solution. Typical treatment periods range from 3-24 hours at 37°C. Each investigator should adjust the concentration of Nigericin and treatment period to accommodate the particular cell line and research conditions. See Figures 3-5 for sample data in Nigericin treated THP-1 monocytes and Jurkat cells.
   - **Danger:** Nigericin is toxic if swallowed, causes skin irritation, causes serious eye irritation, and may cause respiratory irritation. 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.

11. PREPARATION OF FLICA

FLICA is supplied as a lyophilized powder that is dried onto the base of the amber glass vial. It 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.

1. Reconstitute each vial of FLICA with 50 µL DMSO to form the 150-300X stock concentrate. The stock solution should appear as a clear, blue-green solution. 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 30-60X FLICA solution. Use 30-60X FLICA within 30 minutes of dilution into aqueous buffers.

12. PREPARATION OF 1X CELLULAR WASH BUFFER

ICT's Cellular Wash Buffer (catalog #6164) 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 Cellular Wash Buffer contains 0.01% w/v sodium azide). Alternative solutions, including cell culture media containing FBS and other additives may be used to wash and stabilize cells instead of 1X Cellular Wash Buffer.

1. 10X Cellular 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 Cellular Wash Buffer 1:10 in diH_2O. For example, add 15 mL 10X Cellular Wash Buffer to 135 mL diH_2O for a total of 150 mL.
   - 1X Cellular Wash Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

13. PREPARATION OF HOECHST 33342

Hoechst 33342 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. It can be used with FLICA to label the nuclei of live, dying, and apoptotic cells. To use, add to samples at 0.5% v/v and incubate 10-20 minutes at 37°C. For example, if the cell suspension or overlay medium volume is at 300 µL, add 1.5 µL Hoechst 33342.

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 which is below the threshold for reporting on the 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 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.

Fixative will not interfere with the FLICA 660 label. If using absolute ethanol or methanol-based fixatives, caution is recommended as they have been shown to inhibit the fluorescence output of other fluorescent labels, such as carboxyfluorescein, and may affect the fluorescence potential of the FLICA 660 label.

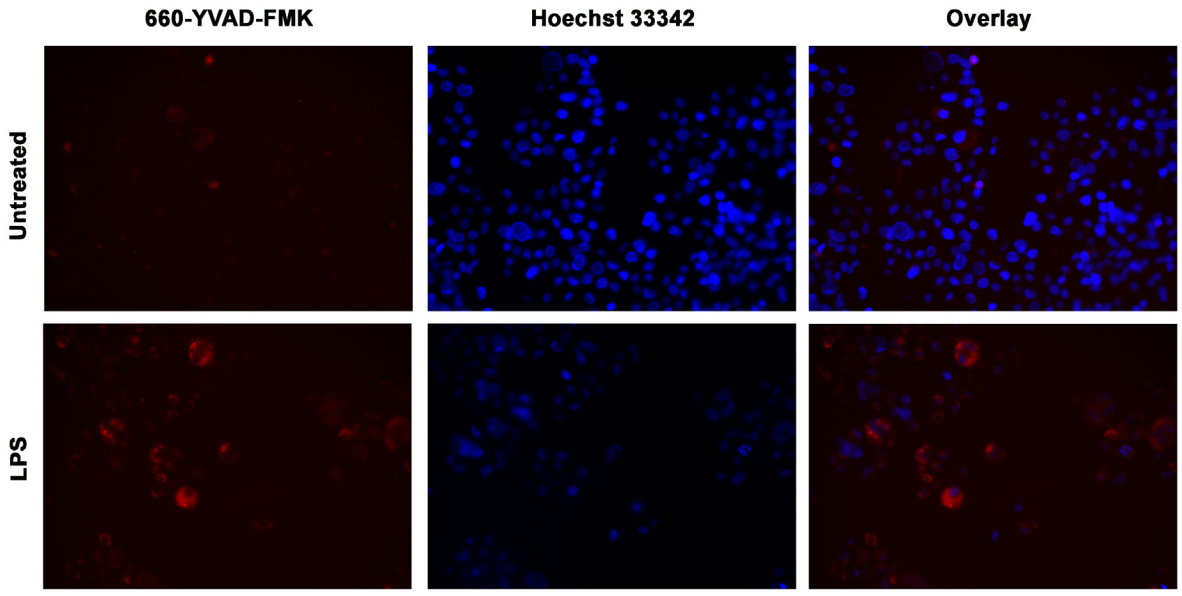
- **Danger:** Fixative contains formaldehyde <10% and methanol <5% and is harmful. Avoid contact with skin, eyes, and clothing by wearing lab coat, gloves, and safety glasses. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

15. CELL STAINING PROTOCOL

Prepare experimental and control cell populations. Ideally, the 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

FIGURE 2: FLUORESCENCE MICROSCOPY IMAGING OF THP-1 CELLS

Intracellular caspase-1 activity was detected in THP-1 cells using ICT's active caspase-1 reagent, 660-YVAD-FMK. THP-1 cells were seeded onto chamber slides and were then treated with PMA (5 ng/mL for 48 hours) to become macrophage-like. After 48 hours, media containing PMA was replaced with fresh media, and cells were allowed to recover for 4 days, and then were treated with LPS (100 ng/mL for 1 hour, lower row of images), or were untreated (upper row of images), and were then immediately stained with 660-YVAD-FMK for 1 hour at 37°C (concurrent with treatment). Following FLICA staining, samples were washed, stained with Hoechst 33342 for 15 minutes at room temperature, and imaged. Intracellular caspase-1 activity was detected using a Logos iRiS Digital Cell Imaging System equipped with Cy5 (Ex 620/60, Em 700/75) and DAPI (Ex 375/28, Em 460/50) LED filter cubes at 20X. The images below show untreated cells (upper row of images) and LPS-treated cells (lower row of images) stained with 660-YVAD-FMK (leftmost images), Hoechst 33342 (middle images), or an overlay of both the Cy5 and DAPI channels (rightmost images). Data courtesy of Mrs. Tracy Murphy (ICT 230:32).



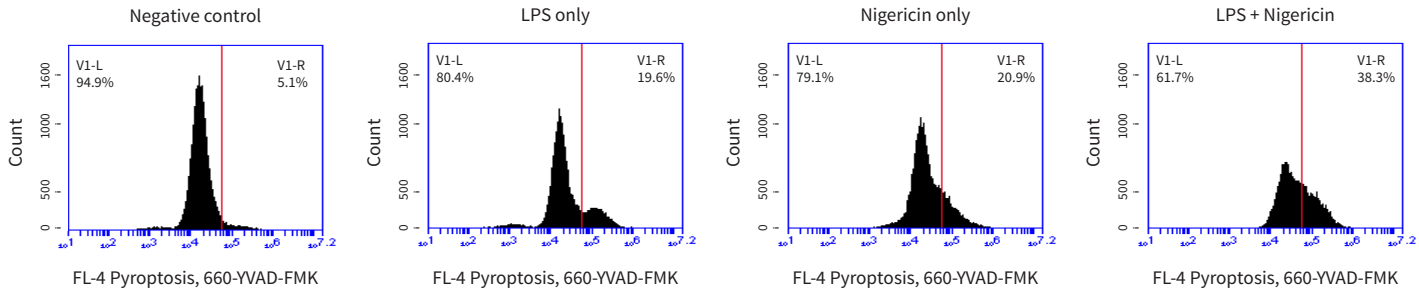
prior to staining with FLICA, cells may need to be concentrated to 2-5 x 10^6 cells/mL as microscopy normally requires higher cell concentrations (Section 16). Start with a larger volume of cells at 3-5 x 10^5 cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 µL per sample when ready for FLICA staining.

When working with adherent cells, care is recommended during handling to avoid the loss of any cells that round up and come off the culture surface. In microscopy applications where trypsinization is not required, adherent cells can be stained and washed directly on the chamber slide, well, coverslip, 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. If suspension cells are required, such as in flow cytometry applications, 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. Expose cells to the experimental and control conditions.
   a. If analyzing with a flow cytometer, set aside two populations to create instrument controls with FLICA. Positive and negative control cells induced and not induced to activate caspase-1 (Section 8).
   b. If analyzing with a fluorescence microscope, 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 efficiently analyze samples at 3-5 x 10^5 cells/mL.
2. Transfer 290-295 µL cells into fresh tubes. If staining adherent cells, FLICA can be added directly to the overlay medium.
3. Add 5-10 µL 30-60X FLICA 660 working 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 gently. The concentration 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 applications.
4. 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. In suspension cell cultures, cells may settle on the bottom of the tubes; gently resuspend by swirling cells every 20 minutes to ensure an even distribution of FLICA.
5. Wash cells. For suspension cells, go to Step 6. For adherent cells, go to Step 7.
6. Wash suspension cells:
   a. Add 2 mL 1X Cellular 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 analysis, repeat wash procedure a third time (resuspend samples, gently pellet by centrifugation, and carefully remove supernatants). Go to Step 8.
7. 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 FLICA and

FIGURE 3: CASPASE-1 ACTIVITY IN THP-1 MONOCYTES

ICT's pyroptosis detection reagent, 660-YVAD-FMK was used to monitor the caspase-1 induction response in THP-1 cells treated with LPS (1 µg/mL for 3 hours), Nigericin (20 µM for 30 minutes), or LPS + Nigericin (1 µg/mL LPS for 3 hours, followed by 20 µM Nigericin for 30 minutes). A common cell pool was spiked with 660-YVAD-FMK and divided into the following treatment groups: 1.) Negative control, 2.) LPS only, 3.) Nigericin only, and 4.) LPS + Nigericin. LPS was added to "LPS only" and "LPS + Nigericin" samples at 1 µg/mL and the samples were incubated at 37°C in a cell culture incubator. After 3 hours, Nigericin was added to "Nigericin only" and "LPS + Nigericin" treatment groups. Samples were returned to the 37°C cell culture incubator for 30 additional minutes. Following their respective treatments, cells were washed and analyzed on an Accuri C6 flow cytometer. Samples treated with either LPS or Nigericin alone had approximately 4 times the level of caspase-1 activity compared to the negative control sample. The sample treated with both LPS and Nigericin had nearly twice the level of caspase-1 activity as either treatment alone. Data courtesy of Mrs. Tracy Murphy, ICT (220:95).



replace with 1X Cellular Wash Buffer.

b. Incubate 10 minutes at 37°C to allow any unbound FLICA to diffuse out of cells.

c. Carefully remove and replace overlay wash buffer with fresh wash buffer and incubate another 10 minutes at 37°C.

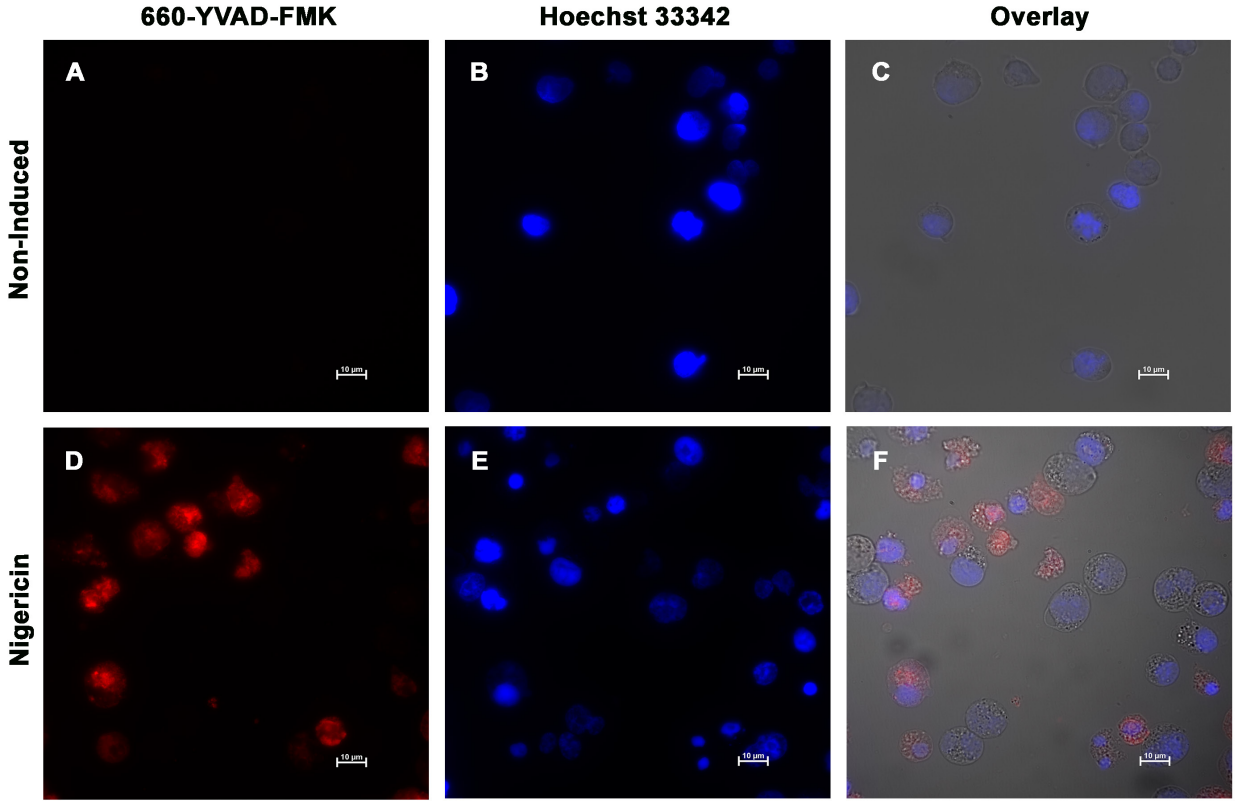
d. Gently remove overlay wash buffer and replace for a third wash step. Incubate 10 minutes at 37°C.

e. Gently remove overlay wash buffer. Go to Step 8.

8. If using a microscope, cells may be counter-stained with ancillary dyes like the nuclear stain Hoechst 33342 or other compatible fluorescent dye. Do not stain with Hoechst if using a flow cytometer (Section 17).
   - Resuspend cells or replace overlay 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 300 µL, add 1.5 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 instructions for staining samples.
9. Read cells within 4 hours or fix.
   - If using a fluorescence microscope, go to Section 16.
   - If using a flow cytometer, go to Section 17.

FIGURE 4: FLUORESCENCE MICROSCOPY IMAGING OF JURKAT CELLS

Jurkat cells were mock treated with a negative control (Non-Induced, Panels A-C), or Nigericin (10 µM) to induce NLRP3 inflammasome and caspase-1 activation (Nigericin, Panels D-F). Samples were then immediately stained with 660-YVAD-FMK, therefore FLICA reagent was present throughout the induction process. Following addition of FLICA, the cells were incubated for 24 hours at 37°C, washed, stained with Hoechst 33342 for 15 minutes at room temperature (to label nuclei blue), and examined under a Nikon Eclipse 90i fluorescence microscope equipped with a Hamamatsu Flash 4.0 camera. In the non-induced sample, many cells with blue nuclei are visible in Panel B. However, in Panel A, which shows 660-YVAD-FMK labeling, no red cells with active casapse-1 are visible. Panel C shows the overlay image combining the blue and red fluorescence channels with the corresponding differential interference contract (DIC) image, which reveals cell morphology. In the Nigericin treated sample, many cells with blue nuclei are visible in Panel E, the majority of which are labeled red in Panel D, indicating the presence of active caspase-1 enzymes. Panel F shows the image made by overlaying blue fluorescence, red fluorescence, and DIC channels into a single combined image. Data courtesy of Mrs. Tracy Murphy, ICT (220:98).



16. MICROSCOPY ANALYSIS

Follow Section 15.

1. Resuspend cells or replace overlay medium with 300-500 µL 1X Cellular 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.
   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. For adherent cells grown on a coverslip, mount the coverslip with cells facing down onto a drop of PBS. If a chamber slide was used, pull off the plastic frame and add a drop of PBS to the cell surface and cover with a coverslip.
4. Observe cells with a fluorescence microscope using a bandpass filter (excitation 660 nm, emission >680 nm) to view far red fluorescence. Hoechst 33342 can be detected using a UV-filter with excitation at 365 nm and emission at 480 nm.

17. FLOW CYTOMETRY ANALYSIS

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

1. Resuspend cells in 300 µL 1X Cellular 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 controls. 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 visualized.
4. For single-color analysis, a 633 nm helium-neon laser or comparable >640 nm laser illumination source can be used. Measure FLICA 660 emission on the FL-4 channel or with emission filters compatible with light emission between 680-690 nm.
5. Run single color controls. Generate a histogram with the log FL-4 on the x-axis versus the number of cells on the y-axis. Caspase negative (FLICA 660-) cells will fall within the lower log fluorescence output decades of the FL-4 x-axis, whereas caspase-positive (FLICA 660+) cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram. Adjust the voltage on FL-4, 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 the experimental samples and analyze.

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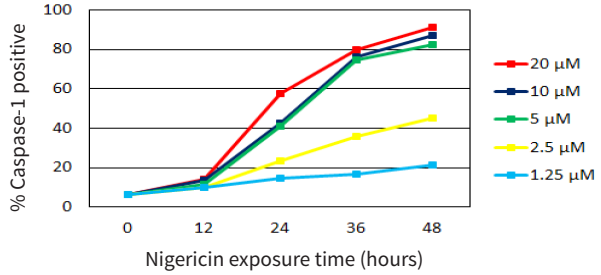
FIGURE 5: CASPASE-1 ACTIVITY IN JURKAT CELLS

ICT's caspase-1 inhibitor reagent, 660-YVAD-FMK was used to monitor the caspase-1 induction response in Jurkat cells treated with Nigericin for various periods of time. A common cell pool was spiked with 660-YVAD-FMK and divided into separate treatment groups. Samples were treated with 1.25, 2.5, 5, 10, and 20 µM concentrations of Nigericin and incubated at 37°C for 0, 12, 24, 36, and 48 hours. Following exposure to Nigericin, the cells were washed and analyzed on an Accuri C6 flow cytometer. The amount of caspase-1 activity detected correlated to both the duration of exposure and the Nigericin concentration; the longer the cells were exposed to Nigericin and the higher the concentration, the larger the proportion of caspase-1 positive cells found in the sample. Data courtesy of Mrs. Tracy Murphy, ICT (230:01).



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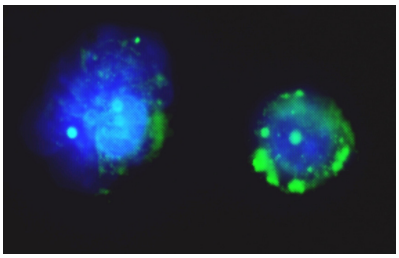

Above: Apoptotic neuroblastoma cells fluoresce green after staining with FAM-FLICA® Poly Caspase Assay (#92). Hoechst 33342 (blue in image) nucleic acid stain is included in the kit as well as Propidium Iodide live/dead stain (not shown).

At left: SR-FLICA® Poly Caspase Assay, Standard Size (#917)

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ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.

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