FLISP® Serine Protease Assays

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

Assess serine protease activity in whole cells in vitro using FLISP.

1. INTRODUCTION

ImmunoChemistry Technologies' (ICT's) FLISP® serine protease assays enable researchers to detect intracellular chymotrypsin-like serine protease activity *in vitro* without lysing the cell. FLISP® (Fluorescent Labeled Inhibitors of Serine Proteases) reagents are non-cytotoxic green or red fluorescent inhibitors that covalently bind with active serine protease enzymes.

FLISP is a powerful method to assess the intracellular levels of chymotrypsin-like serine protease activity *in vitro*. Just add FLISP directly to the cell culture media, incubate, and wash. Because FLISP inhibitors are cell-permeant, they will efficiently diffuse in and out of all living cells. If there is an active chymotrypsin-like enzyme inside the cell, it will covalently bind with the FLISP inhibitor and retain the green or red fluorescent signal within the cell. Cells containing lower concentrations of chymotrypsin-like enzyme activity will retain a lower level of fluorescence compared to cells containing higher concentrations of this effector enzyme. There is no interference from pro-enzymes nor inactive forms of the enzymes. If the treatment is activating chymotrypsin-like serine proteases, positive cells will fluoresce brighter than the normal baseline negative cells, thus enabling researchers to clearly differentiate the populations.

Serine proteases are a family of proteolytic enzymes defined by the presence of a serine residue at the active center of the enzyme, which participates in the formation of an intermediate ester to transiently form an acyl-enzyme complex[1]. The most characterized enzymes of this type are trypsin and chymotrypsin. All living cells have a base level of chymotrypsin-like enzymatic activity which will vary with the physiological state of the cell as well as by cell type. Activated serine proteases play major roles in several different functions including: apoptosis[2]; markers of tumor malignancy[3-5]; diagnostic and prognostic indicators of breast carcinomas[6,7] and neck and head carcinomas[8]. Serine protease activity is also altered in a variety of other cell-mediated diseases related to transplant rejection and infections[9-13].

Because of their supporting role in the apoptotic process, serine protease activity will be greater in apoptotic cell populations compared to healthy cells of the same cell type[14,15]. Activation of caspases is upstream and likely a prerequisite for activation of serine proteases. Using FLISP in combination with FLICA® allows researchers to discriminate serine protease activity from caspase activity in the same cell[14,15] (Figure 2).

Tightly controlled proteolysis is a defining feature of apopotosis[16]. Caspases are critical in this regard but there are also significant roles for non-caspase proteases. These proteases may complement or accelerate caspase-mediated cell death or they may mediate other forms of caspase-independent cell death. Inhibitors of serine proteases can delay cell death in HL-60 cells. Staurosporine activates serine-protease-dependent cell death independently of, but in parallel with, caspase controlled systems in HL-60 cells. A chymotrypsin-like protease is activated during staurosporine-induced apoptosis and appears to be responsible for specific events downstream of mitochondrial disruption in HL-60 cells. Features of the serine protease-mediated cell-death system include cell shrinkage and apoptotic morphology, regulation of caspase-3, altered nuclear morphology, generation of an endonuclease, and DNA degradation[16].

Involvement of serine proteases in apoptosis has been mostly studied by observing whether apoptotic events can be prevented by specific inhibitors of these enzymes. Fragmentation of DNA in HL-60 cells treated with DNA topoisomerase inhibitors to induce apoptosis was prevented by the use of an irreversible serine protease inhibitor, such as N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), which inhibits chymotrypsin[17]. The same inhibitor also inhibited nuclear fragmentation as well as fragmentation of DNA in other cell types, including thymocytes treated with the corticosteroid prednisolone[18,19].

FLISP inhibitors consist of fluorochrome-labeled analogs of the first serine protease inhibitor, tosyl-phenylalanylchloromethyl ketone (TPCK). They are labeled with either a green carboxyfluorescein (FAM) or red sulforhodamine 101 (also known as Texas Red™) fluorochrome, and are available with either a chloromethyl ketone (CMK) or diphenyl 1-(N-peptidylamino) alkane-phosphonate (DAP) reactive-group containing compound[14,15,20-22]. The CMK and DAP reactive groups target the catalytic sites of serine proteases, thus facilitating the covalent labeling of intracellular chymotrypsin-like protease enzymes. CMK-based inhibitors irreversibly alkylate the active site histidine residue of serine proteases[22], while DAP-based inhibitors irreversibly phosphonylate the active site serine residue[20] to give rise to their respective stable inhibitor-enzyme complexes. FLISP inhibitors include: FAM-phenylalanine-CMK (FFCK); FAM-spacer-phenylalanine-CMK (FSFCK); SR101-phenylalanine-CMK (SFCK); FAM-leucine-CMK (FLCK); FAM-spacer-leucine-CMK (FSLCK); SR101-leucine-CMK (SLCK); FAM-phenylalanine-DAP (FFDAP); FAM-leucine-DAP (FLDAP). FFCK with Phe moiety is expected to inhibit chymotrypsin. FLCK with Leu moiety should have a preference to chymotrypsin C[14,15].

Cells labeled with FAM-FLISP can be counter-stained with other reagents such as the red vital stains Propidium Iodide (included in FAM-FLISP Kits) and 7-AAD (Catalog #6163) to identify necrotic cells (Figure 4). Nuclear morphology may be concurrently observed using Hoechst 33342, a blue DNA binding dye (included in all FLISP kits). Cells can be viewed directly through a fluorescence microscope (Figures 2 and 6), or the fluorescence intensity can be quantified using a flow cytometer (Figures 1 and 4) or fluorescence plate reader (Figure 3). FAM-FLISP excites at 488-492 nm and emits at 515-535 nm. SR101-FLISP excites at 586 nm and emits at 605 nm.

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2. FAM-FLISP KITS (green):

Inhibitor Reagent (Part#)	Kit Catalog # Trial	Standard
FAM-Phe-CMK (FFCK, 6146)	945	946
FAM-Leu-CMK (FLCK, 6148)	949	950
FAM-Spacer-Phe-CMK (FSFCK, 6149)	963	964
FAM-Spacer-Leu-CMK (FSLCK, 6150)	965	966
FAM-Leu-DAP (FLDAP, 6153)	967	968
FAM-Phe-DAP (FFDAP, 6217)	984	985

FAM-FLISP Kits contain:

- Green FAM-FLISP inhibitor reagent:
  1 vial in the Trial Kit; or 4 vials in the Standard Kit
- 10X Cellular Wash Buffer:
  15 mL #6164 in the Trial Kit; or 60 mL #6165 in the Standard Kit
- Fixative (6 mL) #636
- Propidium Iodide, 250 µg/mL (1 mL) #638
- Hoechst 33342, 200 µg/mL (1 mL) #639

3. SR101-FLISP KITS (red):

Inhibitor Reagent (Part#)	Kit Catalog # Trial	Standard
SR101-Phe-CMK (SFCK, 6151)	951	952
SR101-Leu-CMK (SLCK, 6152)	955	956

SR101-FLISP Kits contain:

- Red SR101-FLISP inhibitor reagent:
  1 vial in the Trial Kit; or 4 vials in the Standard Kit
- 10X Cellular Wash Buffer:
  15 mL #6164 in the Trial Kit; or 60 mL #6165 in the Standard Kit
- Fixative (6 mL) #636
- Hoechst 33342, 200 µg/mL (1 mL) #639

4. STORAGE

- Store the unopened FLISP reagent (Pack 1) at ≤-20°C until the expiration date.
- Store the remaining unopened kit components (Pack 2) at 2-8°C until the expiration date.

Once reconstituted with DMSO, use FLISP reagent immediately, or aliquot and store at ≤-20°C for 6 months protected from light. Avoid repeated freeze thaw cycles.

5. SAFETY DATA SHEETS (SDS)

Safety data sheets are available 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 FLISP (Section 11)
- DiH_2O, 135-540 mL to dilute 10X Cellular Wash Buffer (Section 12)
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute FLISP 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 (Section 8)
- Reagents to stimulate serine protease activity or induce apoptosis, such as staurosporine (Catalog #6212) or camptothecin (Catalog #6210), (see Section 10)
- 90% EtOH or 3% formaldehyde to create live/dead controls for Propidium Iodide staining (Section 13)
- Black 96-well microtiter plate, flat bottom, non-treated, non-sterile (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 polystyrene 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-FLISP optimally excites at 488-492 nm and has a peak emission at 515-535 nm.
- SR101-FLISP optimally excites at 586 nm and has peak emission at 605 nm.
- View Propidium Iodide 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 13).
- Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 14).

8. EXPERIMENTAL PREPARATION

Staining cells with FLISP can be completed within a few hours. However, FLISP 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 control process which may require additional incubation. Create cell populations, such as:

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

b. Normal non-treated cells of the same cell line or type that were not exposed to the experimental condition that will act as baseline controls of serine protease enzyme activity.

As FLISP detects serine protease activity, plan the experiment such that FLISP will be diluted and administered at the time when elevated serine protease activity is expected to be evident in the cell treatment population.

The recommended volume of 50X FLISP is 10 µL per 490 µL of cells at 5 x 10^5 cells/mL, but the amount may vary based on the experimental conditions and the instrument used for analysis. Each investigator should adjust the amount of FLISP to accommodate the particular cell line and research conditions.

Culture cells to a density optimal for the specific experiment or control 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 FLISP to use as the resulting positive signal is a direct measurement of chymotrypsin-like enzyme activity occurring during the incubation period.

9. CONTROLS

Establishment of positive and negative cell population controls to assess constitutively expressed cellular house keeping serine proteases can be somewhat challenging. As discussed in Section 8, a normal, non-stimulated cell population control is needed to act as the reference baseline of enzymatic activity. Also, in some cell lines, the addition of conventional apoptosis inducing agents such as camptothecin or staurosporine (Section 10) can generate elevated levels of subsets of the larger chymotrypsin family of serine protease enzymes.

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 serine proteases using a known 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 FLISP and analyzing samples by flow cytometry, create 4 populations:

1&2. Unlabeled: induced and non-induced

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

If using FAM-FLISP inhibitors with Propidium Iodide (PI) vital dye for dual staining, create PI instrument controls using formaldehyde or EtOH (Section 13) to compensate for bleed-over of the PI signal into FAM-FLISP signal (Section 19).

10. STIMULATE SERINE PROTEASE ACTIVITY

Determine a reproducible method for stimulating serine protease activity to obtain a positive control prior to commencing the experiment. Induction of apoptosis may trigger serine protease activity as they are involved in protein degradation along with caspase enzymes (Figures 1, 2, 4, and 6). For example, apoptosis may be induced with:

a. 2-4 µg/mL camptothecin for >4 hours
b. 1-2 µM staurosporine for >4 hours

11. PREPARATION OF FLISP

FLISP 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 50X FLISP staining solution must be used immediately, prepare it just before staining.

1. Reconstitute each vial of FLISP with 50 µL DMSO to form the 250X stock concentrate. The FAM-FLISP stock concentrate should be colorless or light yellow; the SR101-FLISP stock concentrate should be pink or red. Once reconstituted, the stock concentrate 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 the 250X stock concentrate 1:5 by adding 200 µL PBS to each vial to form the 50X FLISP staining solution. Use the 50X staining solution within 15 minutes of dilution into aqueous buffers as the CMK and DAP reactive groups are water labile.

12. PREPARATION OF 1X CELLULAR WASH BUFFER

ICT's Cellular Wash Buffer (Catalog #6164 and #6165) is an isotonic solution used to wash cells following exposure to FLISP. It contains mammalian proteins to stabilize cells stained with FLISP and sodium azide to retard contamination (1X Cellular Wash Buffer contains 0.01% w/v sodium azide). Cell media containing FBS and other additives may be used 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. PROPIDIUM IODIDE

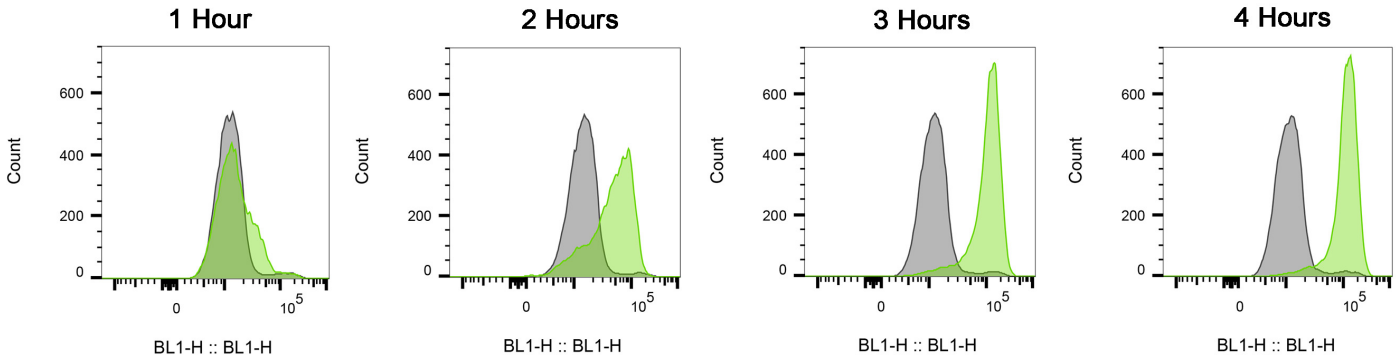
Propidium Iodide (PI, Catalog #638) is used to distinguish between living and dead cells by staining necrotic, dead, and membrane-compromised cells red. PI is an intercalating fluorescent reagent that stoichiometrically binds every four to five base pairs of DNA. PI is membrane impermeant, which prevents 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)

FIGURE 1: DETECTION OF SERINE PROTEASE ACTIVITY DURING 4 HOUR STAUROSPORINE TREATMENT

Intracellular serine protease activity was monitored over time in Jurkat cells exposed to 1 µM staurosporine. Cells were treated with staurosporine for 1, 2, 3, and 4 hours to induce apoptosis and increase serine protease activity (green histograms), or were untreated (gray histograms). Samples were stained with FAM-Phe-CMK (FFCK, Kit #946) for 1 hour at 37°C prior to being washed to remove unbound FLISP and then analyzed using an Attune NxT flow cytometer. The amount of serine protease activity detected correlated to the duration of the exposure period; the longer the cells were exposed to staurosporine, the larger the portion of serine protease positive cells found in the sample. Data courtesy of Dr. Kristi Strandberg (ICT 231:75-79).



which is below the threshold for reporting on the SDS. 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.

All green FAM-FLISP kits include PI in a soluble, ready to use formulation (1 mL at 250 µg/mL). PI is not included in the red SR101-FLISP kits due to the overlap in emissions. If analyzing the cells using flow cytometry (Section 19), establish a PI-positive control population of dead cells to assist with proper compensation. For example, create PI-positive controls using formaldehyde or EtOH to kill cells. A stimulation or apoptosis induction method (Section 10) is not as effective as a solvent treatment (like EtOH) at creating PI-positive cell controls for instrument calibration. 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-stimulated live 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 of PBS to maintain the integrity of the cell membrane. Gently vortex for 30 seconds and then wash cells (Steps 6-8).
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). 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). Gently vortex for 30 seconds and then wash cells (Steps 6-8).
6. Add 1 mL PBS.
7. Centrifuge at 200 x g for 5-10 minutes; remove supernatants.
8. Resuspend in 600 µL PBS+1% BSA.
9. Add 3 µL PI to both tubes. If different volumes were used, add PI at 0.5% v/v.
10. Incubate 5-10 minutes.
11. Read immediately on the flow cytometer (Section 19) and use to compensate bleed over of the PI signal into the FAM channel.

14. 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.

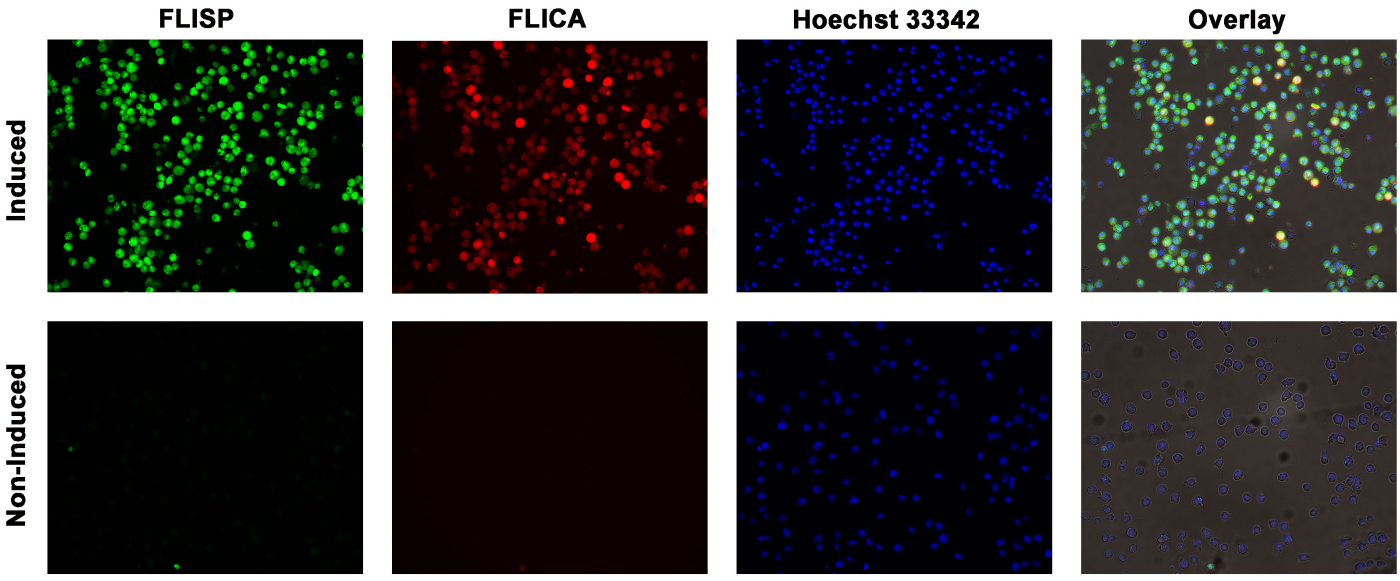
Hoechst 33342 is provided ready to use at 200 µg/mL. Hoechst 33342 can be used with FAM-FLISP, SR101-FLISP, and PI to label cell nuclei. Figure 2 shows cells stained with FAM-FLISP, FLICA 660, and Hoechst 33342.

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 (CAS 23491-52-3) which is below the threshold for reporting. Hoechst 33342 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.

FIGURE 2: DUAL STAINING WITH FAM-FLISP AND FLICA 660

Intracellular serine protease activity was detected in Jurkat cells using ICT's green FAM-FLISP serine protease inhibitor (Kit #946). ICT's far red FLICA 660 poly-caspase inhibitor (Kit #9120) was used to detect caspase activation. Cells were exposed to 1 µM staurosporine for 4 hours to induce apoptosis and increase serine protease activity (upper row of images), or were untreated (lower row of images). During the final hour of treatment, samples were stained with FAM-Phe-CMK and 660-VAD-FMK for 1 hour at 37°C and Hoechst 33342 for 15 minutes at room temperature, and then were imaged. Intracellular localization of the FLISP and FLICA inhibitors, and Hoechst 33342 were detected using a Logos iRiS Digital Cell Imaging System equipped with EGFP (Ex 470/30, Em 530/50), Cy5 (Ex 620/60, Em 700/75), and DAPI (Ex 375/28, Em 460/50) LED filter cubes at 20X. Treatment with staurosporine triggered an increase in the intracellular levels of both serine proteases and caspases compared to non-induced controls. Data courtesy of Dr. Kristi Strandberg (ICT 231:73).



15. 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 FLISP, 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 protected from light on ice or at 2-8°C for up to 16 hours.

ICT's Fixative will not interfere with the carboxyfluorescein (FAM) or sulforhodamine-101 (SR101) label. Do not use absolute ethanol- or methanol-based fixatives as they will inactivate the FAM-FLISP or SR101-FLISP labels. 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.

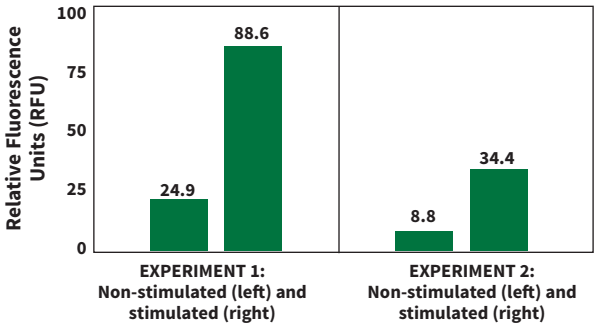
16. STAINING PROTOCOL FOR SUSPENSION CELLS

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 FLISP, cells may need to be concentrated to 2-5 x 10^6 cells/mL as both microscopy and plate reader analysis methods (Sections 18 and 20, respectively) 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 500 µL per sample when ready for FLISP staining.

1. Expose cells to the experimental or control condition. If analyzing with a flow cytometer, set aside the appropriate single-color cell populations to create instrument controls (FLISP-induced and FLISP-non-induced cells, and if necessary, PI-positive and PI-negative cells).
2. If analyzing with a fluorescence microscope or plate reader, concentrate cells to 2-5 x 10^6 cells/mL just prior to FLISP 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 490 µL cells into fresh tubes.
4. Add 10 µL 50X FLISP staining solution (Section 11) and gently mix. If different cell volumes were used, add 50X FLISP staining solution at a ratio of 1:50. Mix by gently flicking the tubes. The amount of FLISP 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 FLISP.
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 RT or 37°C. For example, if the cell suspension is at 500 µL, add 2.5 µL Hoechst. Omit this step if cells are to be analyzed by flow cytometry or fluorescence plate reader.
7. Add 2 mL 1X Cellular 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 Cellular 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.
13. Read cells within 4 hours or fix. Fixed cells may be stored protected from light on ice or at 2-8°C for up to 16 hours.
    - If analyzing with a fluorescence microscope, go to Section 18.
    - If using a flow cytometer, go to Section 19.
    - If using a fluorescence plate reader, go to Section 20.

FIGURE 3: SERINE PROTEASE ACTIVITY QUANTIFIED WITH A FLUORESCENCE PLATE READER

Relative serine protease activity was measured using a Molecular Devices Gemini SpectraMax fluorescence plate reader. Jurkat cells were treated with a non-stimulating control or with an agent to stimulate serine protease activity. Cells were then labeled with ICT's green FAM-FLISP serine protease inhibitor reagent FFCK (Kit #946) and analyzed. The experiment was done twice. Although there is a significant difference in brightness intensity as measured in relative fluorescence units (RFU) between the 2 experiments, the ratio of non-stimulated:stimulated RFU was almost the same in each sample. The stimulated population of cells in Experiment 1 had a high signal of 88.6, while the stimulated population in Experiment 2 was 34.4 which is closer to the non-stimulated population from Experiment 1 at 24.9. The ratio of non-stimulated:stimulated RFU in Experiment 1 was 24.9:88.6=1:3.6, and Experiment 2 was higher at 8.8:34.4=1:3.9. Data courtesy of Ms. Tracy Hanson (ICT 200:73).



17. 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 FLISP before or after trypsinization. Do NOT trypsinize 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 T25 flasks, culture dishes, or chamber slides and expose to the experimental or control conditions.
2. If suspension cells are required for the final analysis, go to Step 3. If staining cells while adherent, go to Step 5.
3. Trypsinize adherent cells:
   a. Remove overlay media. Spin to pellet loose cells.
   b. Trypsinize adherent cells. Alternatively, FLISP labeling can be per-

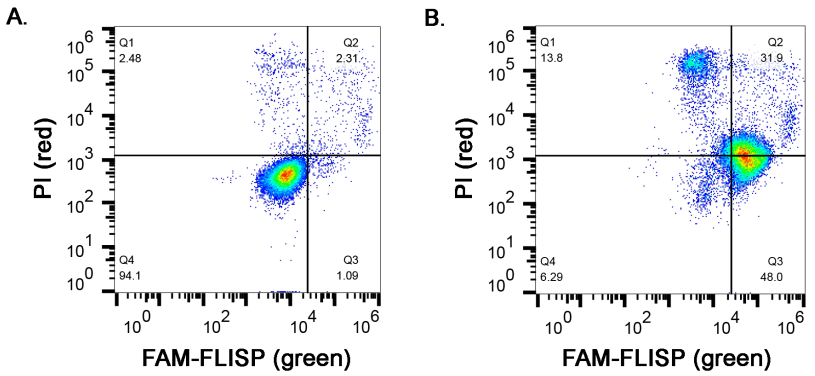
formed first, followed by washing and trypsinization steps.

c. Neutralize with 2-5 mL of a trypsin inhibitor, such as cell culture media containing 20% FBS.

4. Adjust volume of trypsinized cell samples 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 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.
5. Add the 50X FLISP working solution to samples at a v/v ratio of 1:50. Mix the cell suspension to disperse the FLISP reagent. If staining adherent cells, add FLISP directly to the overlay media. The concentration of FLISP should be optimized for each cell line, experimental condition, and method of analysis.
6. Incubate cells at 37°C protected from light, mixing gently every 10-20 minutes to disperse the reagent. The incubation period may range from 30 minutes to several hours and should be optimized for each cell line and experimental condition.
7. Wash cells. For trypsinized adherent (suspension) cells, go to Step 8 or for adherent cells, go to Step 9.
8. Wash trypsinized adherent (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 or fluorescence plate reader analysis, repeat wash procedure a third time by following steps d-f.
   h. Cells may be counter-stained with ancillary dyes like the nuclear stain Hoechst 33342 (go to Step 10) or other compatible dyes, or cells are ready for analysis (go to Step 11).
9. 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 FLISP and replace with 1X Cellular Wash Buffer.
   b. Incubate 10 minutes at 37°C to allow any unbound FLISP to diffuse out of cells.
   c. Carefully remove and replace wash buffer with fresh wash buffer.
   d. Incubate 10 minutes at 37°C.
   e. Gently remove buffer and replace for a third wash step. Incubate 10 minutes at 37°C.
   f. Gently remove buffer. Cells may be counter-stained with ancillary dyes like the nuclear stain Hoechst 33342 (go to Step 10) or other compatible dyes, or cells are ready for analysis (go to Step 11).
10. To stain samples with Hoechst 33342 or other compatible fluorescent dyes:
   a. Resuspend cells or replace overlay buffer.
   b. Add Hoechst 33342 at 0.5% v/v, and incubate 10-20 minutes at RT or 37°C. For example, if the cell suspension or overlay volume is at 1 mL, add 5 µL Hoechst 33342.
   c. If using other compatible ancillary dyes, follow the manufacturer's instructions for staining samples.
   • 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 FLISP 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.
11. Read cells within 4 hours or fix. Fixed cells may be stored protected from light on ice or at 2-8°C for up to 16 hours.
   • If analyzing with a fluorescence microscope, go to Section 18.
   • If using a flow cytometer, go to Section 19.
   • If using a fluorescence plate reader, go to Section 20.

FIGURE 4: DUAL STAINING WITH FAM-FLISP AND PROPIDIUM IODIDE

Intracellular serine protease activity was detected in Jurkat cells using ICT's green FAM-FLISP serine protease inhibitor FLCK (Kit #950) and cells with compromised membrane activity were identified using Propidium Iodide. Cells were untreated (A.), or were exposed to 1 µM staurosporine for 4 hours to induce apoptosis and increase serine protease activity (B.). During the final hour of treatment, samples were stained with FAM-Leu-CMK for 1 hour at 37°C, and then were washed to remove any unbound FLISP. After wash steps, samples were stained with PI for 15 minutes at room temperature, and then were analyzed using an Attune NxT flow cytometer. Staurosporine treatment resulted in an increase in the median fluorescence intensity for both FLISP (BL1-H, x-axis) and PI (YL1-H, y-axis). Data courtesy of Dr. Kristi Strandberg (ICT 231:80-81).



18. MICROSCOPY ANALYSIS

Follow Section 16 or 17.

1. Resuspend cells or replace overlay media in 300-500 µL 1X Cellular 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 2.5 µL PI to the 500 µL cell suspension. If different volumes were used, add it at 0.5% v/v. Incubate 5 minutes at 37°C.
   a. Wash cells to remove excess PI from the media. Centrifuge at 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 Cellular Wash Buffer and gently mix. Go to Step 3 or 4.

3. If not viewing immediately, cells may be fixed and viewed later. Fixed cells may be stored protected from light on ice or at 2-8°C for up to 16 hours.
   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. Cells bearing active serine protease enzymes covalently coupled to FAM-FLISP appear green, and those coupled to SR101-FLISP appear red.
   a. Observe cells labeled with FAM-FLISP using a bandpass filter with excitation 490 nm and emission >520 nm to view green fluorescence.
   b. View PI (red) under a broad bandpass filter with the excitation at 490 nm, emission >610; optimal settings would be 490 nm excitation and 617 emission; nuclei-bound PI has a maximum emission at 617 nm.
   c. Observe cells labeled with SR101-FLISP using a broad bandpass filter with excitation at 590 nm and emission >610 nm to view red fluorescence.
   d. Hoechst 33342 (blue) can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

19. FLOW CYTOMETRY ANALYSIS

Follow Section 16 or 17, but omit 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 distinguished.
4. For single-color analysis of FAM-FLISP, 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). SR101-FLISP is best analyzed 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.
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. Cells with lower levels of serine protease activity will fall within the lower log fluorescence output decades on the x-axis and the cells with increased levels of serine protease activity will appear as a shoulder or as a separate peak on the right side of the negative peak histogram (Figure 1). Adjust the voltage on the fluorescence channel, if necessary, to ensure fluorescence is on scale and serine protease 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.

FIGURE 5: SERINE PROTEASE INHIBITORS BLOCK ENDOPROTEOLYSIS OF PRELAMIN A BY ZMPSTE24

ICT's serine protease inhibitor FAM-Phe-CMK (FFCK, Kit #946) was used to inhibit the enzyme Zmpste24 (FACE-1). Zmpste24 normally cleaves prelamin A and generates a 31 kDa product. When Zmpste24 was inhibited by FFCK, lower levels of the 31 kDa product were detected. Endoproteolysis reactions were performed for 90 minutes in the absence or presence of a zinc metalloprotease inhibitor [1,10-orthophenanthroline (Opa EDTA)] or serine protease inhibitor (FFCK). The relative formation of the 31 kDa product (as a % of total prelamin A) was quantified using densitometry. Zmpste24 endoproteolysis of prelamin A is blocked by serine protease inhibitors, but not zinc metalloprotease inhibitors. Data courtesy of Douglas P. Corrigan, East Tennessee State University, Johnson City, TN.

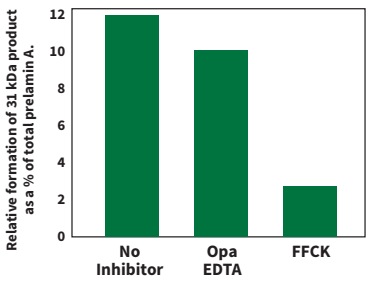
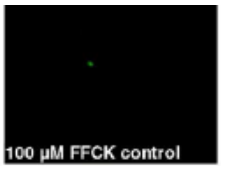
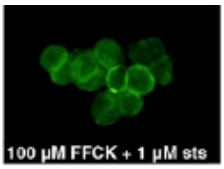


FIGURE 6: STAUROSPORINE ACTIVATES PARALLEL CASCADES OF CASPASE AND SERINE PROTEASE APOPTOSIS

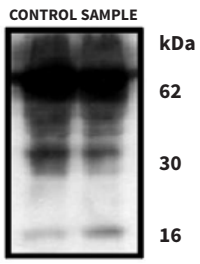
Staurosporine can activate apoptosis even when caspases are inhibited, indicating that other proteases may be involved. ICT's FAM-FLISP FFCK Assay (Kit #946) was used to assess serine protease activity triggered by staurosporine. HL-60 cells were treated with FFCK for 1 hour, then treated with staurosporine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



A: FFCK Control



B: FFCK + Staurosporine



C: FFCK Binding Proteins in Western Blot

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

20. FLUORESCENCE PLATE READER ANALYSIS

Follow Section 16 or 17, 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 (if the induction process caused any cell death), as some 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 ~90% confluency.
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.
   a. For FAM-FLISP: Set the excitation wavelength at 488 nm and the emission wavelength at 530 nm; if possible, use a 515 nm cut-off filter. FAM-FLISP excites at 488-492 nm and the emits at 515-535 nm .
   b. For SR101-FLISP: Set the excitation wavelength at 590 nm and the emission wavelength at 620 nm; if possible, use a 610 nm cut-off filter. SR101-FLISP excites at 586 nm and emits at 605 nm.

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