1. INTRODUCTION

ImmunoChemistry Technologies’ (ICT) FLISP® serine protease detection kits enable researchers to quantitate 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 ICT’s 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 component after the wash step. 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 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. 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; markers of tumor malignancy; diagnostic procedures.

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. The same inhibitor also inhibited nuclear fragmentation as well as fragmentation of DNA in other cell types, including thymocytes treated with the corticosteroid prednisolone.

FLISP® inhibitors consist of fluorochrome-labeled analogs of the first serine protease inhibitor, tosyl-phenylalanine chloromethyl 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. FLISP® inhibitors include: FAM-phenylalanine-CMK (FFCK); FAM-spacer-phenylalanine-CMK (FSDCK); 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, while FLCK with Leu moiety should have a preference to chymotrypsin C. Leu have been shown to inhibit aminopeptidase of Aeromonas.

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. Nuclear morphology may be concurrently observed using Hoechst, 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, 2, and 4) or fluorescence plate reader (Figure 3). FAM-FLISP® excites at 488-492 nm and emits at 515-535 nm. SR-FLISP® excites at 595 nm and emits at 620 nm. FLISP® is for research use only. Not for use in diagnostic procedures.
2. FAM-FLISP® KITS (green):  
**Inhibitor Reagent (part#)** | **Kit Catalog #** | **Small** | **Large**  
--- | --- | --- | ---  
FAM-Phe-CMK (FFCK, 6146) | 945 | 946  
FAM-Leu-CMK (FLCK, 6148) | 949 | 950  
FAM-Spacer-Phe-CMK (FSFCK, 6149) | 963 | 964  
FAM-Leu-DAP (FLDAP, 6153) | 967 | 968  
FAM-Phe-CMK (FFCK, 6146) | 984 | 985  

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

3. SR101-FLISP® KITS (red):  
**Inhibitor Reagent (part#)** | **Kit Catalog #** | **Small** | **Large**  
--- | --- | --- | ---  
SR101-Phe-CMK (SFCK, 6151) | 951 | 952  
SR101-Leu-CMK (SLCK, 6152) | 955 | 956  

**Red SR101-FLISP® kits contain:**  
- Red SR101-FLISP® inhibitor reagent: 1 vial in the small kit; or 4 vials in the large kit  
- Fixative (6 mL) #636  
- 10X Cellular Wash Buffer: 15 mL #6164 in the small kit; or 60 mL #6165 in the large kit  
- Hoechst 33342, 200 μg/mL (1 mL) #639  

4. KIT CONFIGURATION & STORAGE  
Each kit is provided in 2 boxes which are stored at different temperatures:  
- Box 1 contains the FLISP® reagent which is stored at ≤-20°C. Once reconstituted with DMSO, use FLISP® immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.  
- Box 2 contains all the other kit components which is stored at 2-8°C. Do not freeze Box 2. Store each unopened component at 2-8°C until the expiration date.

5. MSDS  
MSDS are available at www.immunochemistry.com.

6. RECOMMENDED MATERIALS  
- DMSO, 50 μL per vial to reconstitute FLISP®  
- DH2O, 135-540 mL to dilute Cellular Wash Buffer  
- 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 or tissues treated with the experimental conditions ready to be labeled  
- Reagents to stimulate serine protease activity or induce apoptosis, such as staurosporine (catalog #6212) or camptothecin (catalog #6210)  
- 90% ETOH (in 10% PBS or 1X Cellular Wash Buffer) to create PI controls  
- Hemocytometer  
- Centrifuge at 200 g  
- 15 mL polystyrene centrifuge tubes (1 per sample)

7. DETECTION EQUIPMENT  
FAM-FLISP® excites at 488-492 nm and emits at 515-535 nm. SR101-FLISP® excites at 595 nm and emits >620 nm. View Propidium Iodide (PI) under a long pass filter with the excitation at 490 nm, emission >520 nm; nuclei-bound PI has a maximum emission at 617 nm (Section 13). Hoechst stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 14). Use filter pairings that best approximate these settings.  
- Fluorescence microscope  
- Fluorescence plate reader  
- Flow cytometer

8. EXPERIMENTAL PREPARATION  
Staining apoptotic cells with 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:  
- Cells that were exposed to the experimental condition, stimulation, or treatment  
- 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 chymotrypsin-like 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 with FLISP®.

9. CONTROLS  
Establishment of positive and negative cell population controls to assess constitutively expressed cellular housekeeping 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 enzyme 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. In all cases however, the control cells should be of the same cellular composition as the experimental treatment populations being examined. Create non-stimulated controls by culturing an equal volume of cells for every labeling condition. 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 with FLISP®.

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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 2 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 very fine 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® stock concentrate 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 and 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 FLISP® 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 used to wash cells. 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 may be used instead of 1X Cellular Wash Buffer to wash cells.

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 diH₂O. For example, add 15 mL 10X Cellular Wash Buffer to 135 mL diH₂O 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 VITAL DYE

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

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 dye 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. PI efficiently excites at 488-492 despite the fact that its optimal excitation when bound to DNA is 535 nm. In its DNA-bound form, PI emits maximally at 617 nm. This excitation and emission spectra allow for efficient analysis using fluorescence microscopy or flow cytometry. All green FAM-FLISP® kits include PI in a solubilized, 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), it is often useful to establish a PI-positive control population for proper gating of PI-positive cells in FL-3 emission regions if there is any concern of red fluorescence emission overlap into the green emission zone of the FAM-FLISP® probes. Here is a simple protocol to create PI-positive controls using ETOH to kill cells instead of an apoptosis-induction protocol using staurosporine or camptothecin (Section 10). A stimulation or apoptosis induction method is not as effective as a solvent treatment (like ETOH) at creating PI-positive cell controls for instrument calibration.

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**RESEARCH USE ONLY**

**NOT FOR DIAGNOSTIC PROCEDURES**

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**FIGURE 1: FLOW CYTOMETRY ANALYSIS**

ICT’s green FAM-FLISP® reagent, FAM-Phe-CMK (catalog #946), was used to assess chymotrypsin-like serine protease activity in Jurkat cells. Cells were grown in normal cell culture media (RPMI 1640 +10% FBS) and exposed to a control or 1 μM staurosporine (catalog #6212) to stimulate intracellular levels of serine proteases for 4 hours at 37°C. All experimental cells were stained with FAM-Phe-CMK, washed 3 times in 1X Cellular Wash Buffer, stained with Propidium Iodide (PI), a red vital stain, and analyzed using two-color flow cytometry. Additional cell populations of non-FLISP® stained non-stimulated and stimulated Jurkat cells were set aside for gating of the cells using forward-scatter (FSC) and side-scatter (SS) analysis (A). On the dot plots, green fluorescence from serine protease activity is measured in FL-1 on the X-axis, and red fluorescence from necrosis is measured in FL-2 on the Y-axis. FAM-FLISP® + PI stained, non-stimulated (B) and stimulated (C) Jurkat cell dot plots illustrate an increase in staurosporine-stimulated chymotrypsin-like protease activity via a rightward shift in overall cell population green fluorescence output (C).
Induced samples will not have enough late-stage apoptotic cells that have become membrane-compromised to stain positive for PI. It is more effective to compensate using controls that are essentially either all PI-positive (>90%) or all PI-negative.

1. Label 2 centrifuge tubes:
   a. PI-negative
   b. PI-positive
2. Add 1-5 x 10^4 non-stimulated live healthy cells to each tube.
3. Centrifuge at 200 x g for 5 minutes at room temperature (RT) to pellet cells; remove supernatants.
4. To create the PI-negative control and keep the cells alive, resuspend the cells in 300 μL of PBS+1% BSA to maintain the integrity of the cell membrane.
5. To create the PI-positive control and kill most of the cells, resuspend these cells in 300 μL of 90% ETOH in PBS which may be made by adding 9 mL ETOH to 1 mL PBS.
6. Gently vortex each tube for 30 seconds.
7. Add 1 mL PBS+1% BSA.
8. Centrifuge at 200 x g for 5 minutes; remove supernatants.
9. Resuspend in 600 μL PBS+1% BSA.
10. Add 3 μL PI to both tubes. If different volumes were used, add PI at 0.5% v/v.
11. Incubate 5-10 minutes.
12. Read immediately on the flow cytometer (Section 19).

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

Hoechst 33342 (catalog #639) is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used to stain the nuclei of living or fixed cells blue and is often used to distinguish condensed, pyknotic nuclei in apoptotic cells. When bound to nucleic acids, the maximum absorption is 350 nm, and its maximum emission is 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm. Hoechst stain may be used with FAM-FLISP®, SR101-FLISP®, and PI.

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

ICT’s Fixative (catalog #636) is a formaldehyde solution designed to cross-link intracellular components. It will not interfere with the carboxyfluorescein (FAM) or sulforhodamine-101 (SR101) label, unlike the use of absolute ethanol- or methanol-based fixatives, which will inactivate the FAM-FLISP® and SR101-FLISP® labels. If the stained cell populations cannot be evaluated immediately after labeling with FLISP®, add Fixative at a ratio of 1:10. For example, add 100 μL Fixative to 900 μL cells. Do not fix cells that will be stained with Propidium Iodide or Hoechst Stain. Never add Fixative until the staining and final wash steps have been completed. Fixed cells may be stored protected from light on ice or at 4°C up to 24 hours.

16. STAINING PROTOCOL FOR SUSPENSION CELLS
1. Prepare experimental and control cell populations. Cells should be 3-5 x 10^6 cells/mL (which is a typical density for cell culture) but should not exceed 10^6 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis. It may be necessary to start with a larger volume of cells, then concentrate the cells and resuspend to 500 μL when ready for FLISP® staining.
2. Expose cells to the experimental or control condition.
3. Just prior to staining with FLISP®, cells may need to be concentrated 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.
4. Transfer 500 μL cells into fresh tubes.
5. Add 10 μL FLISP® staining solution (Section 11) and gently mix. If different cell volumes were used, add 50X FLISP® staining solution at a ratio of approximately 1:50. The amount of FLISP® should be optimized for each cell line and experimental condition.

FIGURE 2: DUAL STAINING WITH FAM-FLISP® AND SR-FLICA®
HL-60 cells were treated with camptothecin (catalog #6210), an apoptosis inducer, for 3 hours then stained with ICT’s green FAM-FLISP® serine protease inhibitor FFCK (kit #946) and ICT’s red SR-FLICA® poly-caspase inhibitor SR-VAD-FMK (kit #917) for 1 hour. Cells were washed then analyzed on a scanning laser cytometer. Cells with active serine proteases stain green with FFCK along the Y-axis and cells with active caspases stain red with SR-VAD-FMK along the X-axis. Co-localization of serine protease activity versus caspase activity is evident in dually stained cells (B, C, D, and G). The light-scatter image (F) reveals many negative cells. In this experiment, the treatment triggered both caspase activity and serine protease activity. Activation of caspases was rapidly followed by serine protease activation and the time between was relatively short as very few cells are just red (E). Data courtesy of Dr. Jerzy Grabarek, Brander Cancer Center, NY, and Pomeranian School of Medicine, Szczecin, Poland.
6. 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.

7. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells every 20 minutes to ensure an even distribution of FLISP®.

8. If cells are to be analyzed with a microscope, cells may be dually stained with Hoechst:
   a. Add 2.5 μL Hoechst per 500 μL sample. If different volumes were used, add it at 0.5% v/v.
   b. Incubate 5 minutes at room temperature (RT) or 37°C.

9. Wash cells to remove any unbound FLISP® and excess Hoechst (if used) from the media:
   a. Add 2 mL 1X Cellular Wash Buffer and gently mix.
   b. Incubate 5 minutes at RT or 37°C.
   c. Centrifuge at 200 x g for 5 minutes at RT.
   d. Carefully remove and discard the supernatant. Gently vortex the pellets to disrupt clumping.

10. Wash cells again:
    a. Add 1 mL 1X Cellular Wash Buffer and gently mix to resuspend cells.
    b. Incubate 5 minutes at RT or 37°C.
    c. Centrifuge at 200 x g for 5 minutes at RT.
    d. Carefully remove and discard the supernatant. Gently vortex pellets to disrupt clumping.

11. Add 300-500 μL 1X Cellular Wash Buffer to resuspend cells and place on ice. At this point, the cells may be stained with PI for bicolor analysis (Step 12), fixed for future viewing (Step 13), or observed immediately (Step 14).

12. If using green FAM-FLISP®, dead cells may be identified by dual staining with red PI:
    a. Add 1.5 μL PI to 300 μL cell suspension. If different volumes were used, add it at 0.5% v/v.
    b. Incubate 5 minutes at RT or 37°C.
    c. Wash cells to remove excess PI from the media: centrifuge at 200 x g for 5 minutes at RT.
    d. Carefully remove and discard the supernatant. Gently vortex the pellets to disrupt clumping.
    e. Add 300 μL 1X Cellular Wash Buffer and gently mix to resuspend cells. Go to Step 13, 14 or 15.

13. If not viewing immediately, cells may be fixed for viewing up to 24 hours later. Do not fix cells that were treated with Hoechst:
    a. Add 30 μL Fixative. If cells were resuspended in a different volume, add Fixative at a ratio of 1:10.
    b. Incubate 15 minutes at RT in the dark.
    c. Place cells onto a microscope slide and let dry.
    d. Briefly rinse cells with PBS.
    e. Cover cells with mounting media and coverslip.
    f. Store slides at 2-8°C up to 24 hours.

17. MICROSCOPY ANALYSIS OF SUSPENSION CELLS

Follow Section 16, Steps 1-10.

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 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 34.4:8.8=1:3.9. Data courtesy of Ms. Tracy Hanson, ICT 200:73.

Jurkat suspension cells were treated with DMSO, a negative control (A), or an agent to stimulate serine protease activity, (B), then incubated with ICT’s green FLISP serine protease inhibitor reagent, washed, and analyzed with a flow cytometer. The negative control treatment exhibited serine protease activity in only 13.5% of the cell population (A: M2), whereas the experimental treatment stimulated serine protease activity in 56.3% of the experimental cells (B: M2). This is a ratio of 1:4. Data courtesy of Dr. Brian W. Lee & Sally Hed, ICT.
14. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.

   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 stain (blue) can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

18. SINGLE COLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

Follow Section 16, Steps 1-10, but omit Hoechst staining. See Figures 1, 2, and 4.

11. Add 300 μL 1X Cellular Wash Buffer to resuspend cells, and place on ice.

12. For single-color analysis, use a 15 mW argon laser at 488 nm for green FAM-FLISP™. If labeling with red SR101-FLISP™, set the excitation at 488 nm and use FL-2 emission settings.
   a. Set the acquisition template to FW vs. SS density or dot plot.
   b. Run the non-stimulated, non-stimulated control cells and draw a gate around the target population.
   c. Run the non-stimulated, FLISP®-stained negative control cells and generate a histogram with the log FL-1 on the X-axis versus the number of cells on the Y-axis.
   d. Run the positive control stimulated FLISP®-stained cells and confirm that the FLISP®-labeled cells have shifted to the right along the FL-1 axis relative to the negative control. If needed, adjust the voltage/gain settings so that negative (FLISP®-)cells will occur in the lower log fluorescence output decades of the FL-1 (X) axis, whereas serine protease-positive (FLISP®+) cells will appear as a shoulder on the right side (brighter) or a separate peak on the right side of the negative peak histogram.
   e. Set markers so the flow cytometer can calculate percentages of FLISP®-positive cells (Figures 1 and 4).

19. MULTICOLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

Dual staining studies using other reagents labeled with a red dye may be performed using green FAM-FLISP™ inhibitors, but not with red SR101-FLISP™ inhibitors, which are often incompatible.

To address compensation issues and set up the flow cytometer, prepare four instrument controls:

1&2. PI-labeled: live and killed cells that are only stained with PI (Section 13)

3&4. FAM-FLISP™-labeled: stimulated and non-stimulated cells that are only stained with green FAM-FLISP™.

15. Perform an endpoint read.
   a. For green FAM-FLISP™, set the excitation wavelength to 488 nm and the emission wavelength to 530 nm and use a cut-off filter at 515 nm.
   b. For red SR101-FLISP™, set the excitation wavelength to 590 nm and the emission wavelength to 620 nm and use a cut-off filter at 610 nm (Figure 3).
21. STAINING PROTOCOL FOR ADHERENT CELLS
Adherent cells may be labeled with FLISP® and analyzed, or labeled before or after trypsinization and treated as suspension cells (Section 22), or grown and labeled in a microtiter plate (Section 23). Adherent cells need to be carefully washed to avoid the loss of any cells that round up and come off the plate surface. Loose cells may be harvested from the plate or slide surface and treated as suspension cells, while those remaining adherent to the surface should be treated as adherent cells. If the adherent cells are trypsinized, the loose cells can be re-combined with the trypsinized pool; alternatively, the loose cells can be recombined with the adherent portion when the analysis is performed. If growing adherent cells in a plate, the entire plate may be gently spun as part of the wash process to sediment any loose floating cells. Avoid trypsinizing cells prior to labeling with a vital dye, like PI, as cell membranes exposed to trypsin could become transiently permeant to vital dyes.
1. Expose adherent cells to the experimental conditions and create positive and negative controls.
2. Dilute the 50X FLISP® staining solution at approximately 1:50 in cell culture media (Section 11). For example, add 10 μL 50X FLISP® staining solution to 500 μL media.
3. Add enough FLISP® staining solution to completely cover the cell monolayer. Optimize the amount of FLISP® for each cell type and experimental condition.
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.
5. Gently mix by swirling the media every 20 minutes to ensure an even distribution of FLISP®
6. If cells are to be labeled with Hoechst stain:
   a. Add 2.5 μL Hoechst per 500 μL sample. If different volumes were used, add it at 0.5% v/v.
   b. Incubate 5 minutes at RT or 37°C.
7. Carefully remove the overlay medium (minimize the number of adherent cells displaced) and discard. Keep any loose cells and add them back for analysis: spin and wash separately.
8. Wash cells:
   a. Add 1-2 mL 1X Cellular Wash Buffer.
   b. Incubate 5-10 minutes to remove any bound FLISP®
   c. Remove the medium and discard.
9. Wash again:
   a. Add 1-2 mL 1X Cellular Wash Buffer.
   b. Incubate 15-30 minutes.
   c. Remove the medium and discard.
10. Add 1X Cellular Wash Buffer.
11. If cells are to be labeled with PI:
   a. Add 2 μL PI and mix.
   b. Rinse cells with 1X Cellular Wash Buffer to remove any excess PI, add a coverslip, and put samples on ice.
   c. Analyze PI-stained cells as quickly as possible (within 30 minutes) as cells may begin to have toxicity issues.
12. If cells cannot be analyzed immediately, they may be fixed. Do not fix cells if they are counterstained with Hoechst or PI.
   a. Dilute Fixative 1:10 v/v with 1X Cellular Wash Buffer.
   b. Place one drop of diluted Fixative onto the cell surface followed by a cover slip.
   c. Fixed slides may be stored at 2-8°C protected from light up to 24 hours.
13. Add a coverslip and view under a fluorescence microscope (Section 17 Step 15).

FIGURE 5: SERINE PROTEASE INHIBITORS BLOCK ENDOPROTEOLYSIS OF PRELAMIN A BY ZMPSTE24
ICT’s serine protease inhibitor FAM-Phe-CMK (FFCK, catalog #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) 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 APOTOPSIS IN HL-60 CELLS
Staurosporine can activate apoptosis even when caspases are inhibited, indicating that other proteases may be involved. ICT’s FAM-FLISP® FFCK kit (catalog #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 analyzed via Western blotting using anti-fluorescence 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.
22. STAINING AND TRYPsinIZATION PROTOCOL FOR ADHERENT CELLS

Follow Section 21, Steps 1-8.
9. Rinse adherent cells with PBS and keep detached cells. Spin down detached cells and set aside for recombination with trypsin disassociated cells.
10. Trypsinize adherent cells to disassociate cell monolayers.
11. Neutralize detached trypsinized cells by removing them from the plate or flask and adding them to cell culture media containing 20% FBS. Use at least 4-5 times the volume of media+20% FBS to assure complete trypsin neutralization.
12. Pool trypsinized cells with the supernatant cells.
13. Wash cells 3 times:
   a. Centrifuge at 200 x g for 5 minutes at RT.
   b. Carefully aspirate the supernatant and discard.
   c. Add 1-2 mL 1X Cellular Wash Buffer.
   d. Incubate 5 minutes at RT or 37°C.
   e. Centrifuge at 200 x g for 5 minutes at RT.
   f. Aspirate the supernatant and discard.
   g. Resuspend in 1-2 mL 1X Cellular Wash Buffer.
   h. Incubate 5 minutes at RT or 37°C.
   i. Centrifuge at 200 x g for 5 minutes at RT.
   j. Aspirate the supernatant and discard.
14. Resuspend the cell pellet in 500 μL PBS or 1X Cellular Wash Buffer.
15. If cells cannot be analyzed immediately, they may be fixed:
   a. Add 50 μL 10X Fixative to 500 μL cells and mix. If cells were resuspended in a different volume, add 10X Fixative at a 1:10 v/v dilution.
   b. Fixed cells may be stored at 2-8°C protected from light up to 24 hours.
16. Analyze as suspension cells:
   a. Microscope: Section 17, Step 15.
   b. Flow cytometer: Section 18, Step 12.
   c. Fluorescence plate reader: Section 20, Step 15.

23. FLUORESCENCE PLATE READER ANALYSIS OF ADHERENT CELLS

1. Adherent cells can be grown in a microtiter plate. Use plates with clear bottoms and black walls. Culture cells to approximately 90% confluency.
2. Expose adherent cells to the experimental conditions and create positive and negative controls.
3. Add 50X FLISP® at 1:50 (Section 11).
4. Incubate 30-60 minutes at 37°C, mixing gently every 10 minutes to ensure an even distribution of FLISP®.
5. Add ~300 μL media to each well.
6. Incubate 60 minutes at 37°C to allow any unbound FLISP® to diffuse out of the cells.
7. Gently centrifuge the entire plate to sediment any loose floating cells.
8. Aspirate the media. Resuspend with fresh media or PBS or 1X Cellular Wash Buffer.
9. Read plates using a top-reading instrument (Section 20, Step 15).

24. REFERENCES


Thank you for using FLISP®! If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788 or send an email to help@immunochemistry.com.