SR-FLIVO® *In vivo* Poly Caspase Assay

Catalog #982 & #983

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

Assess poly caspase activity in whole live animals in vivo using SR-FLIVO.

1. INTRODUCTION

FLIVO®(FLuorescence in vIVO) is a powerful method for assessing caspase activity in vivo. Similar to our FLICA® probes[1,2], but optimized for whole live animal imaging, FLIVO probes are non-cytotoxic fluorescent inhibitors of caspases. ICT's SR-FLIVO poly caspase inhibitor probe contains the preferred binding sequence for all caspases, Val-Ala-Asp (VAD). This preferred poly caspase tripeptide binding sequence is labeled at the amino terminus end with a sulforhodamine B (SR) dye and linked at the carboxyl end to a fluoromethyl ketone (FMK) reactive entity. The resulting cell permeant, fluorescent molecule, SR-VAD-FMK, optimally excites at 550-580 nm and emits at 590-600 nm. The spectra for SR-FLIVO is shown in Figure 1.

Apoptosis is an evolutionarily conserved process of programmed cell suicide. It is centered on a cascade of proteolytic enzymes called caspases that are triggered in response to pro-apoptotic signals. Like most other proteases, caspases are synthesized as pro-form precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity[3]. Active caspase enzymes consist of two large (~20 kD) and two small (~10 kD) subunits that non-covalently associate to form a two heterodimer, tetrameric active caspase[4-6]. Once activated, caspases cleave protein substrates leading to the eventual disassembly of the cell. Caspases have been identified in organisms ranging from *C. elegans* to humans. Mammalian caspases play distinct roles in both apoptosis and inflammation.

FLIVO kits provide a simple yet accurate method to detect caspase activity in vivo. To label cells containing elevated levels of active caspases, inject FLIVO intravenously and let it circulate ~60 minutes. Because the reagent is cell-permeant, it readily diffuses in and out of all cells it encounters as it circulates throughout the body. If there are active caspase enzymes inside a cell, FLIVO will form an irreversible covalent bond with a reactive cysteine on the large subunit of the caspase heterodimer, thereby inhibiting further enzymatic activity. The bound FLIVO probe will remain inside the cell as long as the cell membrane is intact. Any unbound FLIVO is removed from the circulation of the animal in about an hour. Additional time may be needed for FLIVO to clear other tissues. The remaining red fluorescent signal in the tissue after unbound FLIVO has cleared is a direct measure of caspase activity that occurred at the time the reagent was injected. Apoptotic cells will retain a higher concentration of FLIVO and fluoresce brighter than non-apoptotic cells. There is no interference from pro-caspases or inactive forms of the enzyme. If the treatment is causing cell death via apoptosis, apoptotic cells will have an elevated level of caspase activity relative to non-apoptotic or negative control cells and fluoresce red with FLIVO. After labeling with FLIVO, excised tissues can be counter-stained with other reagents and fixed or frozen.

Once the animals have been injected with FLIVO and excess unbound FLIVO has cleared from the body of the animal, the tissues are ready for analysis and no further staining is necessary. Because FLIVO is a direct stain, it eliminates any false positives that may arise from manipulation of the tissue. This gives a true representation of the induction of apoptosis in vivo as a result of the experimental condition. Tissues can be viewed directly through a window chamber system or other accessible cavity, or thin tissue sections can be prepared after sacrificing the animal. Tissues labeled with FLIVO can be counter-stained with other reagents such as DAPI and fixed or frozen for future analysis. The fluorescence intensity can be quantified by excising the tissue and analyzing cells with a flow cytometer. SR-FLIVO optimally excites at 550-580 nm and has a peak emission at 590-600 nm (Figure 1).

2. KIT CONTENTS

Small size kits contain:

- 1 vial of SR-FLIVO Poly Caspase Inhibitor (SR-VAD-FMK), 131 µg per vial, #6219
- 1 bottle of 10X Injection Buffer, 5 mL, #6220

Large size kits contain:

- 4 vials of SR-FLIVO Poly Caspase Inhibitor (SR-VAD-FMK), 131 µg per vial, #6219
- 1 bottle of 10X Injection Buffer, 5 mL, #6220

3. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Once reconstituted with DMSO, use FLIVO immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

4. SAFETY DATA SHEETS (SDS)

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

ImmunoChemistry TECHNOLOGIES LLC

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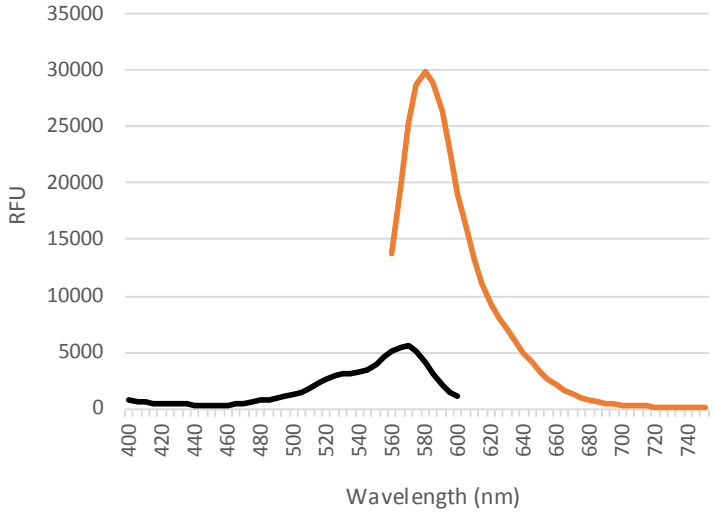
FIGURE 1: SR-FLIVO EXCITATON AND EMISSION SPECTRA

SR-VAD-FMK was reconstituted in DMSO, diluted in an aqueous buffer, and then analyzed on a Molecular Devices M5e plate reader. The excitation spectrum (black) was generated using an emission of 630 nm. The emission spectrum (orange) was generated using an excitation of 540 nm. SR-FLIVO optimally excites at 550-580 nm and has a peak emission at 590-600 nm.



5. RECOMMENDED MATERIALS

- DMSO, 50 µL per vial to reconstitute SR-FLIVO
- DiH_20, 45 mL to dilute 10X Injection Buffer
- 0.2 µm syringe filter to sterilize injection buffer
- Injection materials such as a syringe and needle
- Experimental and control animals ready to be assessed
- Tools to dissect, extract, and examine labeled tissues

6. DETECTION EQUIPMENT

Detection equipment such as a fluorescence microscope, flow cytometer, or window chamber system. SR-FLIVO optimally excites at 550-580 nm and has a peak emission at 590-600 nm.

It may be possible to view SR-FLIVO using instrumentation designed for non-invasive live whole animal imaging, however a final protocol has not yet been established for this technique. The SR fluorophore was found to penetrate up to 3 mm of skin tissue using a Caliper/Xenogen IVIS (see Figure 4).

7. EXPERIMENTAL PREPARATION

Plan your experiment so that FLIVO can be reconstituted, diluted and then injected at the time when caspase activity is expected to be occurring in the animal. It may be necessary to set up an initial experiment to determine when and how much FLIVO to inject as the resulting positive fluorescent signal is a direct measure of caspase activity that occurred at the time of injection. The amount of FLIVO may need to be adjusted to accommodate the particular experimental model and research conditions being investigated.

1. Expose the test animals to your experimental conditions to assess caspase activity in the target tissue(s).
2. Prepare the animals for intravenous injection.

8. PREPARATION OF 1X INJECTION BUFFER

ICT's Injection Buffer is an isotonic solution used for diluting and injecting FLIVO.

1. 10X Injection Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Injection Buffer 1:10 in diH_20. For example, add 1 mL 10X Injection Buffer to 9 mL diH_20 for a total of 10 mL.
3. Sterilize the 1X Injection Buffer by filtering through a 0.2 µm syringe filter or equivalent.
    - 1X Injection Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

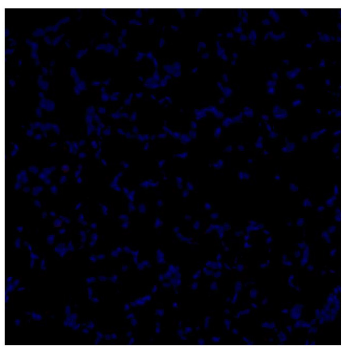
9. PREPARATION OF SR-FLIVO

FLIVO 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 1X FLIVO solution must be used immediately, prepare it just before injection.

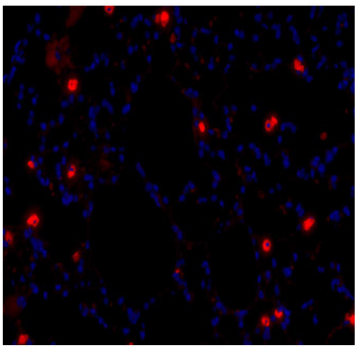
1. Reconstitute each vial of FLIVO with 50 µL DMSO to form the 12X stock. The stock solution should be pink to red in color. Once reconstituted, it may be stored at ≤-20°C for 6

FIGURE 2: DETECTION OF ACTIVATED CASPASES IN MOUSE LUNG TISSUE

Activated caspases were labeled and detected in lung parenchymal cells following whole lung irradiation. Mice were given either a 0Gy or 15Gy whole lung dose of gamma irradiation. Six hours post-irradiation, ICT's SR-FLIVO *in vivo* Poly Caspase Assay (Catalog #983) was injected via mouse tail veins in 100 µl boluses. The 1X FLIVO circulated for 18 hours prior to sacrifice. Mice received an intracardiac perfusion with heparinized saline followed by zinc-buffered formalin. Lungs were inflated prior to removal from the mouse. Tissues were processed, paraffin-embedded and sectioned (5 µm). Nuclei were counterstained with DAPI, then lungs were imaged with a fluorescence microscope. Overlays of SR-FLIVO and DAPI staining are shown below. Active caspases appear as a bright red cytoplasmic stain with discrete blue nuclei. In this model, mice that received 15Gy (right) had greater levels of caspase activity compared to mice that received 0Gy (left). Data courtesy of Eric Hernady (University of Rochester Medical Center, Rochester, NY).



0Gy SR-FLIVO (40x)



15Gy SR-FLIVO (40x)

months protected from light and thawed no more than twice during that time.

2. Immediately prior to injection into the animal, further dilute FLIVO by adding 550 µL sterile 1X Injection Buffer to each vial to form the 1X FLIVO solution. Inject 1X FLIVO within 1 hour of dilution into aqueous buffer; protect from light during handling.

10. INTRAVENOUS INJECTION

1. Inject 100 µL 1X FLIVO into the tail vein or other large vein. Each vial provides enough reagent for 6 animals. If larger injection volumes are needed, dilute FLIVO with more injection buffer and inject 1/6th of the final volume into the animal (approximately 22 µg SR-FLIVO per animal). The exact IV location and amount of FLIVO injected may vary depending on the size of the animal, the target tissue, and the experimental conditions.
2. Allow FLIVO circulate within the animal for 30-60 minutes. After 60 minutes, most of the unbound FLIVO will have cleared from the bloodstream. However, additional time may be needed for FLIVO to clear other tissues. FLIVO is cleared from the animal via the kidneys and liver. Generally, the longer the reagent circulates, the lower the background signal; however, some positive cells (apoptotic or pyroptotic) may be lost over time. FLIVO will remain inside a cell containing active caspases as long as the cell membrane is intact. The optimal duration for FLIVO circulation may need to be determined experimentally.

11. PREPARATION OF TISSUES

Prepare the animal tissues according to your desired protocol. Several methods are listed below.

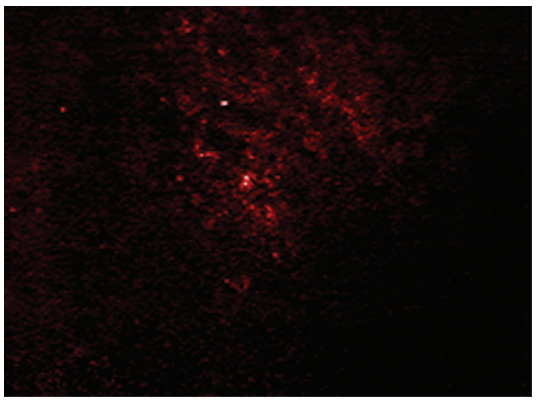
- Perfuse the animal and sacrifice
- Excise the tissue, freeze, and make thin tissue sections
- Extract the tissue and disassociate cells for flow cytometric analysis
- View through a window chamber system

12. REFERENCES

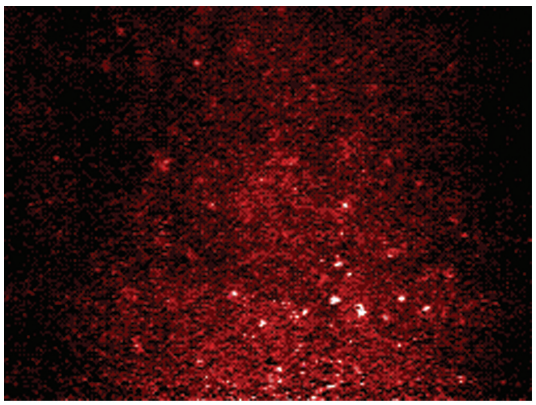
1. Amstad, P. A. et al. Detection of caspase activation in situ by fluorochrome-labeled caspase inhibitors. Biotechniques 31, 608-610, 612, 614, passim (2001).
2. Bedner, E., Smolewski, P., Amstad, P. & Darzynkiewicz, Z. Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. Exp Cell Res 259, 308-313, doi:10.1006/excr.2000.4955 (2000).
3. Kumar, S. Mechanisms mediating caspase activation in cell death. Cell Death Differ 6, 1060-1066, doi:10.1038/sj.cdd.4400600 (1999).
4. Wilson, K. P. et al. Structure and mechanism of interleukin-1 beta converting enzyme. Nature 370, 270-275, doi:10.1038/370270a0 (1994).
5. Walker, N. P. et al. Crystal structure of the cysteine protease interleukin-1 beta-converting enzyme: a (p20/p10)2 homodimer. Cell 78, 343-352 (1994).
6. Rotonda, J. et al. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol 3, 619-625 (1996).

FIGURE 3: *IN VIVO* APOPTOSIS DETECTION

SR-FLIVO was used to assess the effectiveness of arsenic trioxide (ATO) treatment on SCK mammary tumors *in vivo*. Using a window chamber to view tumors directly, SCK mammary tumor cells were grown under the skin of A/J mice for 7 days. Test mice were treated with ATO and the control mice received a placebo (24 hours). All mice were injected IV with SR-FLIVO and images were captured 30 minutes later. The control SCK tumor (left) exhibited a base level of apoptosis as expected (18%, FACS data not shown), while the ATO-treated tumor (right) exhibited a much higher level of apoptosis (36%, FACS data not shown). Bright spots indicate high levels of caspase activity within the tumor. ATO treatment doubled the level of caspase activity. Data courtesy of Dr. Robert Griffin, University of Minnesota.



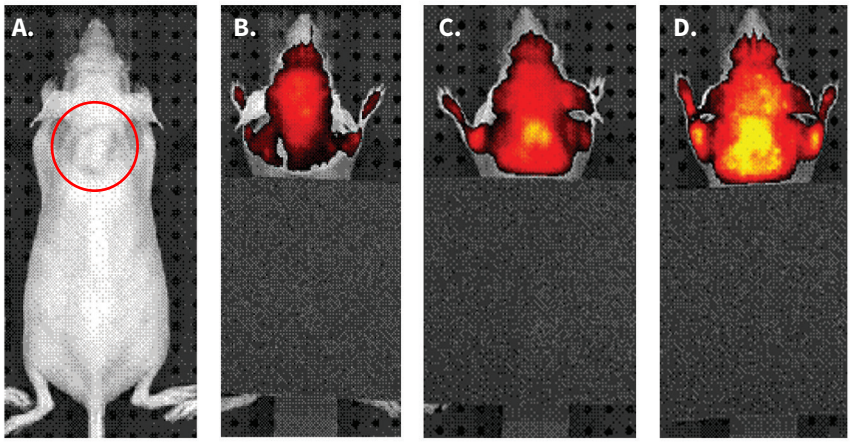
PLACEBO-TREATED TUMOR



ATO-TREATED TUMOR

FIGURE 4. NON-INVASIVE IMAGING OF APOPTOSIS

SR-FLIVO was used for non-invasive imaging of apoptosis in live animals. Human colon carcinoma COLO205 cells were injected S/C into female nude mice (A, tumor circled). After 27 days, animals were treated with a control (B) or TRAIL at 25 mg/Kg (C and D), then injected with SR-FLIVO to image caspase-positive tumor cells. TRAIL induced apoptosis within 1 hour post-treatment (C), and the level of apoptosis significantly increased by 4 hours (D). Data courtesy of Dr. Peter Lassota, Caliper Life Sciences / Xenogen.



Tumor site | Control 4 hours | 25 mg/kg TRAIL 1 hour | 25 mg/kg TRAIL 4 hours

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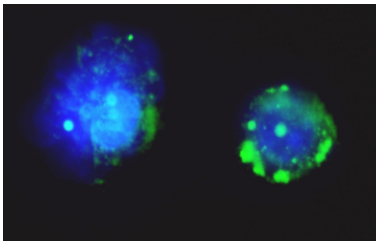
RELATED PRODUCTS:

To detect the intracellular process of apoptosis via activated caspases *in vitro*, use our FLICA® kits. These caspase assays are available in green, red, or far red fluorescence for the preferred detection of individual active caspase enzymes or pan-caspase activity.

Visit www.immunochemistry.com for more information.

- Label active caspases
- Distinguish apoptosis from necrosis
- Whole cell analysis



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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BRIGHT MINDS, BRIGHT SOLUTIONS.

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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