FAM-FLIVO® *In vivo* Poly Caspase Assay

Catalog #980 & #981

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

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 use in whole live animals, FLIVO probes are non-cytotoxic fluorescent inhibitors of caspases. ICT's FAM-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 carboxyfluorescein (FAM) dye and linked at the carboxyl end to a fluoromethyl ketone (FMK) reactive entity. The resulting cell permeant, fluorescent molecule, FAM-VAD-FMK, excites at 488-492 nm and has a peak emission at 515-535 nm. The spectra for FAM-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 *Caenorhabditis 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 that 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 green 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 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 (Figure 6) or other accessible cavity, or thin tissue sections can be prepared after sacrificing the animal (Figure 2). Tissues labeled with FLIVO can be counter-stained with other reagents such as red Nissl (Figure 3) or blue 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 (Figure 4). FAM-FLIVO excites at 488-492 nm and has a peak emission at 515-535 nm.

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

2. KIT CONTENTS

Small size kits contain:

- 1 vial of FAM-FLIVO Poly Caspase inhibitor (FAM-VAD-FMK), 52 µg per vial, #6218
- 1 bottle of 10X Injection Buffer, 5 mL, #6220

Large size kits contain:

- 4 vials of FAM-FLIVO Poly Caspase inhibitor (FAM-VAD-FMK), 52 µg per vial, #6218
- 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 FAM-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.

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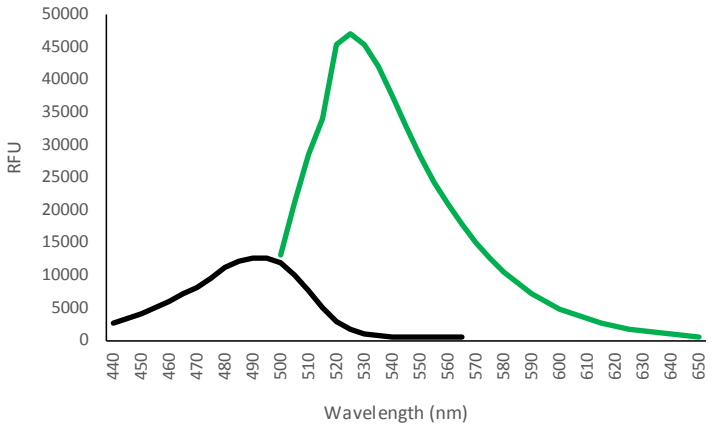
FIGURE 1: FAM-FLIVO EXCITATON AND EMISSION SPECTRA

FAM-FLIVO Poly Caspase Inhibitor 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 590 nm. The emission spectrum (green) was generated using an excitation of 460 nm.



5. RECOMMENDED MATERIALS

- DMSO, 50 µL per vial to reconstitute FAM-FLIVO
- Di$H_2$0, 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

Tissues can be viewed directly through a window chamber system or other accessible cavity. Alternatively, tissues can be excised and cells can be analyzed by fluorescence microscopy, flow cytometry, or fluorescence plate reader. FAM-FLIVO optimally excites at 488-492 nm and has a peak emission at 515-535 nm.

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 di$H_2$0. For example, add 1 mL 10X Injection Buffer to 9 mL di$H_2$0 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.

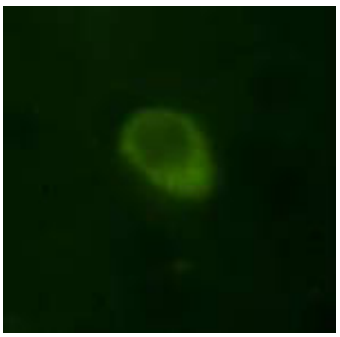
9. PREPARATION OF FAM-FLIVO

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 colorless to light yellow. Once reconstituted, it may be stored at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
2. Immediately prior to 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.

FIGURE 2: APOPTOSIS IN CHICK BRAIN

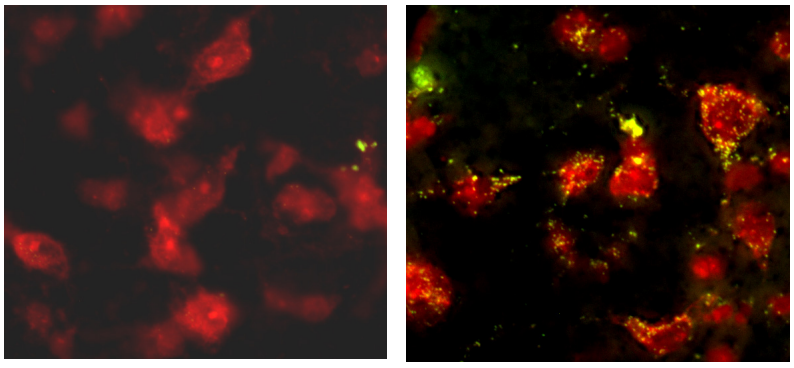
As part of his thesis work to examine naturally-occurring neuron death in seasonally-manipulated songbirds, Mr. Chris Thompson at the University of Washington, Seattle used FAM-FLIVO (catalog #981) to assess apoptosis *in vivo*. Mr. Thompson injected staurosporine (catalog #6212, a protein kinase inhibitor that induces apoptosis) into the forebrain of a female house sparrow. Approximately 20 hours later, he injected FAM-FLIVO intravenously into the jugular and let it circulate 30 minutes. He then sacrificed the bird via transcardial perfusion with heparinized saline and 4% paraformaldehyde. He post-fixed the brain for 48 hours, embedded it in gelatin, and cryoprotected it in 10% NBF and 20% sucrose for 48 hours more. 40 µm slices of the brain were made on a freezing microtome, and sections were mounted onto slides and coverslipped with ProLong antifade mountant. Neurons with active caspases fluoresce green. This image shows one apoptotic neuron at 100X.



APOPTOTIC NEURON IN SPARROW BRAIN

FIGURE 3: CASPASE ACTIVITY IN RAT BRAIN

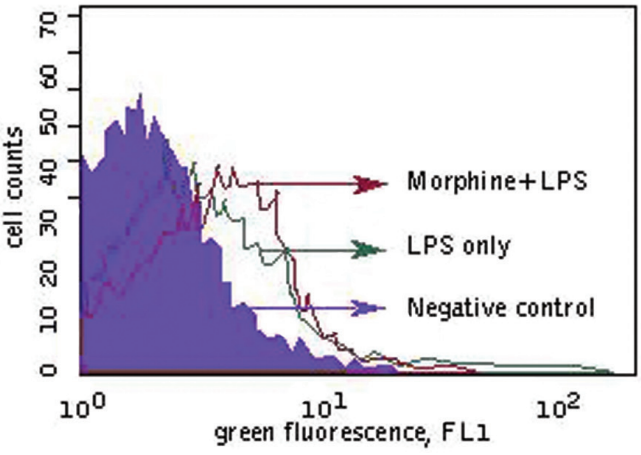
Using FAM-FLIVO to monitor cell death, there is a clear distinction between healthy and apoptotic neurons. In this live animal brain study of diabetes, Dr. Thomas Morrow at the University of Michigan VAMC Ann Arbor was able to assess neurodegeneration via caspase activity in control (left) and 8-week STZ diabetic rats (right). 30 minutes prior to sacrifice, FAM-FLIVO (catalog #981) was injected intravenously to directly label caspase-positive apoptotic neurons. After sacrifice, 20 µm frozen sections of the periaqueductal gray (PAG) were prepared and counter-stained with red fluorescent Nissl to identify all neurons. Dying apoptotic neurons exhibit dual staining with FAM-FLIVO (yellow/green) and Nissl (red). In this model, diabetic animals show greater levels of caspase activity in the PAG than control animals.



CONTROL RAT DIABETIC RAT

FIGURE 4: APOPTOSIS INDUCTION IN BONE MARROW

Dr. Michael Olin at the University of Minnesota used FAM-FLIVO (catalog #981) to assess apoptosis in bone marrow. C57BL/S126 mice were treated with morphine and/or LPS, or a placebo for 48 hours. FAM-FLIVO was injected in the tail vein 45 minutes prior to sacrifice. Following sacrifice, bone marrow cells were obtained and analyzed by flow cytometry. The data demonstrate an increase in apoptosis in the bone marrow leukocytes of morphine-treated animals that were also exposed to LPS.



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 8.67 µg FAM-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 to 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 (Figure 2)
- Excise the tissue, freeze, and make thin tissue sections (Figures 3)
- Extract the tissue and disassociate cells for flow cytometric analysis (Figure 4)
- View through a window chamber system (Figure 5) or fluorescence microscope.

12. REFERENCES

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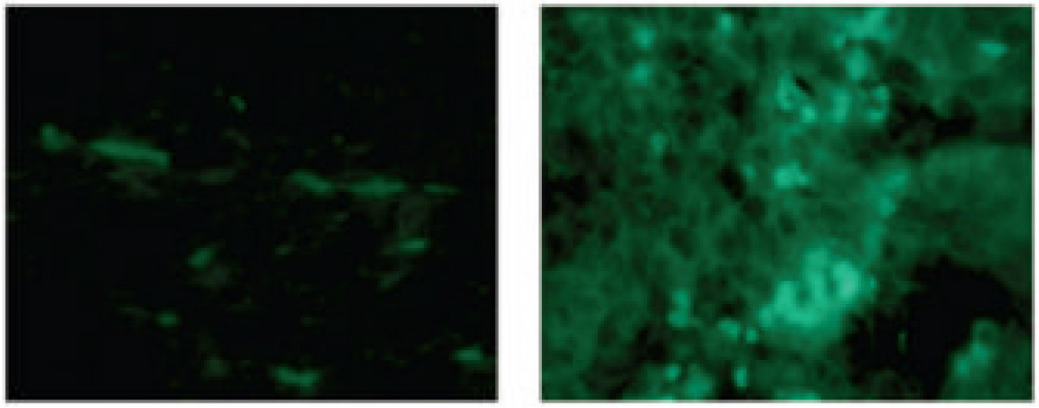
FIGURE 5. *IN VIVO* APOPTOSIS DETECTION

Arsenic trioxide (ATO)-induced apoptosis in FSaII murine fibrosarcoma tumors was detected *in vivo* using FAM-FLIVO. Skin-fold window chambers were surgically implanted into dorsal skin folds on female nu/nu mice. FSaII fibrosarcoma cells were added to skin then glass windows were placed to allow viewing. When tumor growth was apparent, mice were injected with PBS (negative control, left) or 8 mg/kg ATO (apoptosis inducing agent, right). FAM-FLIVO (~10 ug) was injected into each mouse via tail vein 3 hr post ATO injection. Images depict 10X magnification obtained from histological sections of FAM-FLIVO stained FSaII tumors. Data courtesy of R.J. Griffin et. al. Technology in Cancer Research 2007. 6(6) 651-654.



PBS-TREATED TUMOR **ATO-TREATED TUMOR**

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