Exclusively Available from IMGENEX

# pSIVA™-IANBD
## Apoptosis/Viability Flow Kit

**Cat No:** IMG-6700K-100 (100 Test Size)

**Cat No:** IMG-6700K-25 (25 Test Size)

# pSIVA™-IANBD
## Apoptosis/Viability Microscopy Set

**Cat No:** IMG-6701K (Sufficient for 10 ml Staining Solution)

pSIVA™ is a trademark of IMGENEX Corporation

## TABLE OF CONTENTS


I. pSIVA™ Overview . . . . . 3
II. Components and Storage . . . . . 6
- CytoGLO™ pSIVA-IANBD Apoptosis/Viability Flow Kits . . . . . 6
- CytoGLO™ pSIVA-IANBD Apoptosis/Viability Microscopy Set . . 7

III. Features and Advantages . . . . . 8
IV. Applications . . . . . 9
V. pSIVA™ Technology Background . . . . . 9
VI. pSIVA™ Assay Methodology: The Basics . . . . . 10
VII. Flow Cytometry Protocols . . . . . 13
VIII. Microscopy Protocols . . . . . 18
- Basic Fluorescence Microscopy . . . . . 18
- Time-lapse Live Cell Imaging: Introductory Remarks . . . . . 18
  - Time-lapse Imaging: Basic Protocol . . . . . 20
  - Analysis of Viability . . . . . 20
  - Detection of Transient PS Exposure . . . . . 21
  - Analysis of Degeneration and Rescue: Primary Neurons . . 22
    - Neuronal Degeneration Protocol . . . . . 22
    - Neuronal Rescue Protocol . . . . . 24
- *In Vivo* and Tissue Applications . . . . . 26

IX. Frequently Asked Questions . . . . . 27
X. Troubleshooting Guide . . . . . 28
XI. Reporting Cell Death in the Scientific Literature . . . . . 29
XII. General References . . . . . 29
XIII. pSIVA™ Product Citations . . . . . 31


pSIVA™ is a trademark of IMGENEX Corporation

## I. pSIVA™ OVERVIEW

pSIVA (Polarity Sensitive Indicator of Viability & Apoptosis) is an Annexin XII-based, polarity sensitive probe for the spatiotemporal analysis of apoptosis and other forms of cell death (Fig. 1). pSIVA binding is reversible which enables researchers, for the first time, to detect irreversible as well as transient phosphatidylserine (PS) exposure (Fig. 2). PS exposure is a hallmark phenomenon occurring early during apoptosis and persisting throughout the cell death process. PS exposure is most often considered to be irreversible. However, transient PS exposure is increasingly being recognized as a phenomenon in its own right and described to occur during both normal physiological processes and reversible or rescuable apoptotic/cell death events (Bevers et al, 2010; Kim et al 2010a/b).

pSIVA is conjugated to IANBD, a polarity sensitive dye that fluoresces only when pSIVA is bound to the cell membrane (Fig. 3). pSIVA-IANBD fluorescence is measured using conventional FITC filter sets. pSIVA's membrane bound dependent fluorescence and reversible binding properties are a technological leap for detecting PS exposure. These unique properties of pSIVA empower researchers to gain additional information on cell death/cell survival processes compared to

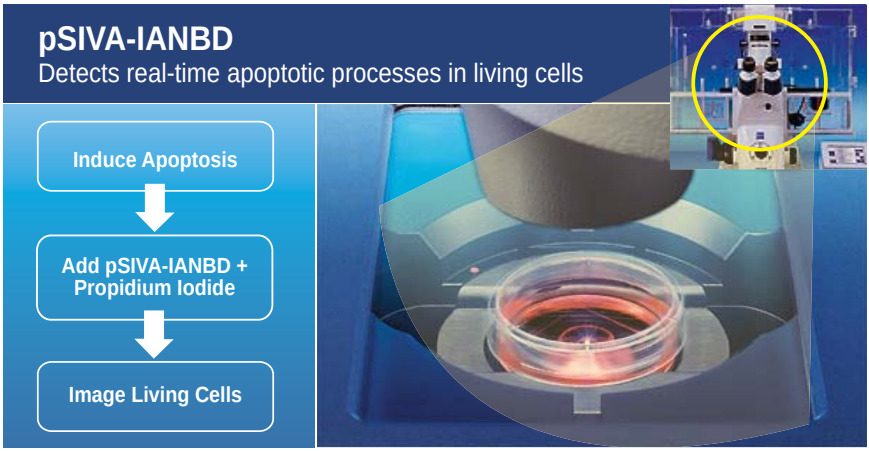


**Figure 1. Monitoring apoptosis real-time in living cells with pSIVA-IANBD.** pSIVA-IANBD binds to cells undergoing apoptosis and can be present in the culture media during the duration of the experiment since it only fluoresces when actually bound to cells. This feature enables time lapsed imaging to monitor the progression of apoptosis and other phenomenon where pSIVA-IANBD binds to cells. pSIVA-IANBD can also be used for flow cytometric, *in vivo* and high throughput applications.

Annexin V conjugates, which fluoresce irrespective of whether or not they are bound to PS. pSIVA-IANBD applications include flow cytometry (IMG-6700K) and live cell fluorescence microscopy imaging (IMG-6701K).

The pSIVA assays are extremely straightforward (Fig. 1): pSIVA-IANBD or pSIVA-IANBD + Propidium Iodide (PI) is directly added to cells or tissues, incubated and analyzed. There are no wash steps! This manual contains detailed information about the pSIVA technology and protocols; please read the manual prior to beginning your experiments!

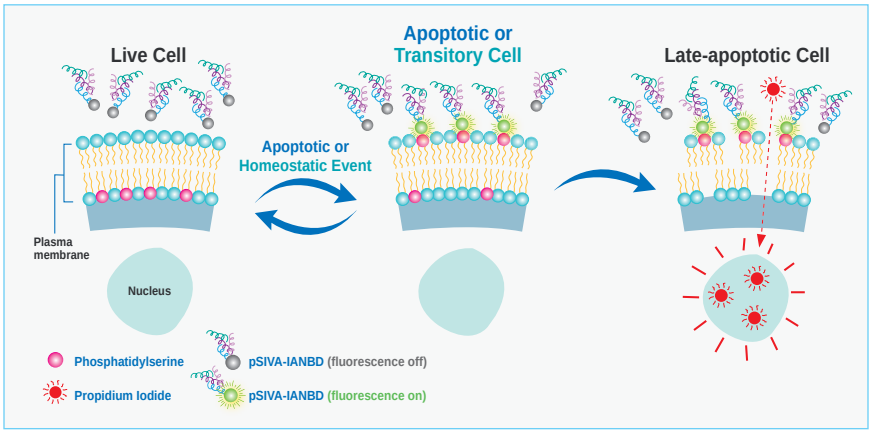


**Figure 2. pSIVA-IANBD: A polarity sensitive, reversible-binding probe for detecting exposed PS residues in the lipid membrane bilayer.** PS translocation from the cytoplasmic to external face of the membrane occurs early in apoptosis and has been considered to be an irreversible event. However, it is now that known that PS exposure may also be ephemeral or reversible. PS flipping back to the cytoplasmic face following homeostatic or rescuable cell death events results in release of PS-bound pSIVA-IANBD and loss of fluorescence. PI is a vital dye used to identify late apoptosis or necrosis which are both associated with loss of membrane integrity.

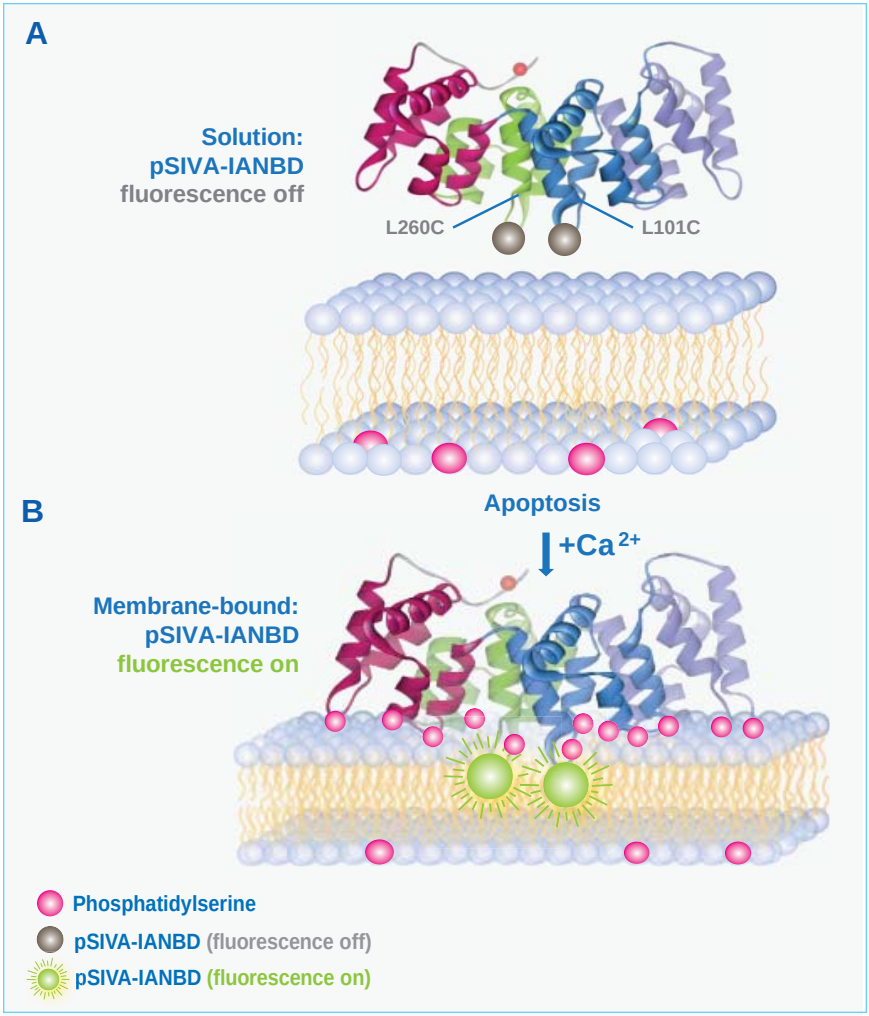


**Figure 3. Structure-based design of pSIVA depicted with the crystal structure of annexin B12 along with the introduced L101 and L260 cysteine (C) mutations.** The cysteine IANBD fluorophore labels are denoted as grey spheres (non-fluorescing) or green stars (fluorescing).
A. PS is primarily localized on the inner leaflet of the plasma membrane of healthy cells, and therefore inaccessible to pSIVA-IANBD. Hence, pSIVA-IANBD remains in solution and the polarity-sensitive IANBD fluorophores do not emit any significant fluorescence (grey spheres).
B. Early during apoptosis, PS translocates to the outer leaflet and becomes accessible to pSIVA. When pSIVA-IANBD bind to PS, the IANBD labels are exposed to the nonpolor lipid environment of the membrane which results in a 'switching on' of the IANBD fluorescence signal. Adapted from Kim et al 2010b.

## II. COMPONENTS AND STORAGE

### CytoGLO™ pSIVA-IANBD Apoptosis/Viability Kits

**Cat No.** IMG-6700K-100 (100 Test Size) Contents*

| Cat No. | Component | Amount | Storage Conditions | Notes |
|---|---|---|---|---|
| KC-146-500 | pSIVA-IANBD | 500 ul | 2-8°C | 5 ul/test (1 x $10^6$ cells) |
| KC-147-500 | Propidium Iodide Staining Solution | 500 ul | 2-8°C | 5 ul/test (1 x $10^6$ cells) |
| KC-148-20 | 10X PBS Buffer | 20 ml | 2-8°C | |
| KC-149-5 | 10X Binding Buffer | 5 ml | 2-8°C | |

**Cat No.** IMG-6700K-25 (25 Test Size) Contents*

| Cat No. | Component | Amount | Storage Conditions | Notes |
|---|---|---|---|---|
| KC-146-125 | pSIVA-IANBD | 125 ul | 2-8°C | 5 ul/test (1 x $10^6$ cells) |
| KC-147-125 | Propidium Iodide Staining Solution | 125 ul | 2-8°C | 5 ul/test (1 x $10^6$ cells) |
| KC-148-5 | 10X PBS Buffer | 5 ml | 2-8°C | |
| KC-149-2 | 10X Binding Buffer | 2 ml | 2-8°C | |

## CytoGLO™ pSIVA-IANBD Apoptosis/Viability Microscopy Set

**Cat No.** IMG-6701K (Sufficient for 10 ml Staining Solution)*

| Cat No. | Component | Amount | Storage Conditions | Notes |
|---|---|---|---|---|
| KC-150 | pSIVA-IANBD | 200 ul | 2-8°C | 10-20 ul/ml |
| KC-147-500 | Propidium Iodide Staining Solution | 500 ul | 2-8°C | 5-10 ul/ml |

***Additional Required Items**

The following are key additional items needed or recommended; they are not included with the Flow Cytometry Kits or Microscopy Set.

- Flow cytometer (IMG-6700K Kits)
- Distilled H20 (IMG-6700K Kits)
- Live-imaging setup (IMG-6701K, see Kim et al, 2010a/b for additional information)
- Experimental cells
- Positive control cell line (recommended, not required). Researchers may want to include a cell line such as Jurkat which is easily induced to undergo apoptosis as a positive control.

## III. FEATURES AND ADVANTAGES

- Advanced PS Exposure Probe
- More applications than Annexin V-conjugates (Table I)
  - Polarity sensitive fluorescence
  - Fluoresces when bound to PS
  - PS binding is reversible
  - Detects both transient and irreversible PS exposure

| Features | pSIVA-IANBD | Annexin V-FITC |
|---|---|---|
| Non-toxic | ✓ | ✓ |
| Detect PS exposure | ✓ | ✓ |
| Detect early apoptosis | ✓ | ✓ |
| Flow cytometry | ✓ | ✓ |
| Viability assessment: Flow cytometry | ✓ | ✓ |
| Toxicity assays | ✓ | ✓ |
| Fluoresces only when bound to PS | ✓ | |
| No washing required | ✓ | |
| Detect transient PS exposure | ✓ | |
| Distinguish between transient & irreversible PS exposure | ✓ | |
| Live-cell imaging | ✓ | |
| Viability assessment: Live-cell imaging | ✓ | |
| In vivo imaging | ✓ | |
| High-throughput screen | ✓ | |

**TABLE I. Comparison of pSIVA-IANBD and Annexin V-FITC.** pSIVA is a new generation Annexin for detecting PS exposure, with more features than Annexin V.

## IV. APPLICATIONS

Applications for pSIVA include flow cytometry (IMG-6700K) and live cell fluorescence microscopy fluorescence imaging (IMG-6701K). pSIVA has been used to analyze PS exposure in various cell lines including Jurkat (Fig 4), HL60 (Fig 5), COS-7 (Kim et al, 2010a), HEK293 (Kim et al, 2010b) and Neuro2A (Zhang et al, 2011) as well as with primary dorsal root ganglion (DRG) neurons (Kim et al, 2010b). Since pSIVA, like Annexin V binds to PS, it should be useful for virtually all the applications, cell types and methods described for Annexin V. However, whereas Annexin V has technical limitations in live cell imaging, pSIVA has been optimized for this technque (Kim et al, 2010a/2010b).

Live-cell imaging is a vital application for studying dynamic biological processes in real time. Synthetic fluorophores and fluorescent proteins are both qualitative and quantitative reporters of intracellular structure and dynamics in live-cell imaging. Heretofore, there has been a lack of available reporters for studying the process of cell death/cell survival in live-cell imaging. Annexin V-conjugates are impractical for live-cell imaging because separate steps are required for binding of the probe and subsequent removal of the unbound Annexin V-conjugate to reduce the background fluorescence before analysis. In contrast, unbound pSIVA-IANBD does not fluoresce and hence there is no washing step nor unwanted background.

pSIVA's most notable live-imaging applications include monitoring the subcellular onset of PS externalization, tracking PS exposure throughout the death process, and demonstrating reversible PS exposure events associated with rescuable cell death (Kim et al, 2010a/2010b). The advent of pSIVA, a novel PS exposure reporter, for live-cell imaging of cell death/cell survival processes may very well be the most significant assay advance in the Cell Death field today.

## V. pSIVA™ TECHNOLOGY BACKGROUND

The pSIVA technology was developed by Ralf Langen from the University of Southern California in 2010, and represents a major advance in cell death assays (Kim et al, 2010a/b). pSIVA was designed by engineering cysteine mutations at residues 101 and 260 in membrane-binding loops of an Annexin B12 plasmid construct, and conjugating the recombinant pSIVA protein to the polar sensitive IANBD dye (Fig. 3). pSIVA-IANBD fluoresces only when bound to PS residues

in the membrane, background fluorescence is minimal. In a landmark series of experiments, the Langen laboratory used pSIVA in a neuronal model system to address some of the most profound unsolved mysteries of cell death (Kim et al, 2010a):

1. Where in the cell is death first evident?
2. Where in a given cell death signaling pathway is the natural point of no return?
3. When in the pathway and where subcellularly can cells no longer be rescued from imminent death?

pSIVA demonstrated that PS exposure, a hallmark of early apoptosis, started in the dendrites prior to proceeding to the cell body. Furthermore, cells were rescuable when evidence of cell death was confined to the dendrites. Rescued cells lost pSIVA-IANBD fluorescence, indicating that PS exposure was not an irreversible event. However, cells could not be rescued when both the dendrites and cell body were pSIVA positive. Hence “Dendrite pSIVA positive” + “Cell body pSIVA positive” denoted a point of no return and a point when PS exposure was no longer reversible.

Historically, cell death assays have been limited in both their ability to pinpoint subcellular death initiation points as well as to identify rescuable or survivable cell death events. pSIVA now enables researchers to address these and potentially other aspects of the cell death/survival process which have heretofore been elusive.

## VI. pSIVA-IANBD ASSAY METHODOLOGY: THE BASICS

The pSIVA-IANBD assay is based on pSIVA, a novel Annexin B12 probe, conjugated to IANBD, a polar sensitive dye to detect the cell surface exposure of PS. pSIVA is a $Ca^{2+}$ dependent binding protein that has high affinity and selectivity for PS, and pSIVA-IANBD fluoresces only when bound to PS (Figs. 2,3). PS on the cell surface is a generally accepted hallmark of cells in apoptosis* and is one of the most widely used markers to detect apoptosis. The detection of PS exposure to track cell death was first established for Annexin V in 1995 by the Reutelingsperger lab (Vermes et al, 1995) and immediately adopted by the Green lab (Green et al, 1995), a preeminent leader in the cell death field. PS exposure

has been cited in thousands of references encompassing a broad spectrum of cell types including a myriad of physiologic and pathologic conditions (PubMed: www.ncbi.nih.gov : search words ‘ Annexin V’, ‘apoptosis’).

In brief, PS is kept localized to the inner leaflet of the plasma membrane by an ATP-dependent aminophospholipid translocase in healthy cells that flips PS from the outer to inner membrane (reviewed in Bevers, 2010). However, PS accumulates by diffusion in the outer leaflet if energy in the form of ATP becomes unavailable or if the plasma membrane loses integrity. In cells undergoing apoptosis, the PS that is normally located on the inner leaflet is translocated to the outer leaflet where it is exposed to the external milieu. Exposure occurs in the early phases of apoptosis while the membrane is still intact, before plasma membrane integrity is lost. PS exposure may parallel caspase-3 activation, another early marker of apoptosis.

PS exposure, however, is not unique to apoptosis but also occurs during other forms of cell death* such as necrosis and even during normal physiological events. Both late stage apoptosis and necrosis are associated with leaky or permeable cell membranes. Hence, pSIVA-IANBD assays can be performed in conjunction with a vital dye such as propidium iodide (PI) to distinguish between non-apoptotic (pSIVA-IANBD negative/PI negative), early apoptotic (pSIVA-IANBD positive/PI negative), and late apoptotic or necrotic cells (pSIVA-IANBD positive/PI positive) (Table II).

| | pSIVA-IANBD | PI |
|---|---|---|
| Healthy cells | - | - |
| Transient PS Exposure | + | - |
| Reversal of PS Exposure | - | - |
| Early Apoptosis | + | - |
| Late Apoptosis | + | + |
| Necrosis | -/+* | + |

**Table II. Tracking Cell Death and PS Exposure Events with pSIVA-IANBD and PI.**
*Should PS flip back from the outer to inner membrane, pSIVA-IANBD-PS binding will be lost and pSIVA-IANBD will be released in the media and no longer fluoresce. Necrosis may happen so quickly that PS binding sites are destroyed or inaccessible due to rapid loss of membrane integrity.

The concept that PS exposure is an irreversible event in the apoptotic program has been a long standing dogma in the cell death field. ***However, pSIVA-IANBD assays have now clearly demonstrated that reversal of PS exposure can parallel rescue of cells from imminent death (Kim et al, 2010a). During rescue pSIVA-IANBD is released from the cell membrane into the external milieu, and PS-bound dependent fluorescence is lost (Table II). PS-bound dependent fluorescence and reversible binding are among the most salient features of pSIVA-IANBD over classical Annexin V-conjugate assays. PS-bound dependent fluorescence can be particularly advantageous in microscopy assays since unbound pSIVA-IANBD lacks fluorescence, and therefore background signal. This is in contrast to Annexin V-fluorescent conjugates which fluoresce irrespective of whether or not they are bound to PS, resulting in background signal and a loss of sensitivity.

As such, pSIVA-IANBD measures exposed PS, and when used in conjunction with PI can track the loss of membrane integrity occurring during the process of cell death. It is important to note that the pSIVA assay, like Annexin V assay, identifies exposed PS and loss of membrane integrity irrespective of the particular term used to describe cell death. Researchers are encouraged to consult the scientific literature for additional information about PS exposure and loss of membrane integrity during the process of various types of cell death.

**Apoptosis is a popular, although loosely defined term that is used differently by different investigators to measure and describe cell death. Other terms used to describe cell death include necrosis, anoikis, caspase-independent apoptosis, autophagy, endoplasmic reticulum stress, Wallerian degeneration, excitotoxicity, eryptosis, mitotic castastrophe, paraptosis, pyroptosis, cornification, pyronecrosis, and entosis (reviewed in Kroemer et al, 2005 and 2009). Researchers should be mindful that cell death assays detect biological or biochemical events which are not necessarily exclusive to only one type of cell death. That is, both pSIVA-IANBD and Annexin V-conjugate assays detect PS exposure rather than detecting 'apoptosis' per se, although PS exposure is considered to be a hallmark of apoptosis.*

****It is interesting to note that in 1999, the Richard Scheuermann laboratory proposed that PS exposure during apoptosis can be reversible and precedes commitment to cell death (Hammill et al, 1999). Flow cytometry assays showed that when the cell death stimulus was removed, there was a notable decrease in Annexin V-FITC positive cells. However, this concept of PS reversibility during apoptosis did not gain widespread attention for more than 10 years, until the advent of pSIVA-IANBD in 2010.*

# VII. FLOW CYTOMETRY PROTOCOLS

## pSIVA™-IANBD Apoptosis/Viability Flow Kit

**Cat No:** IMG-6700K-100 (100 Test Size)
**Cat No:** IMG-6700K-25 (25 Test Size)

**Reagents (root Cat nos. are listed)**

1. pSIVA-IANBD (KC-146): Use 5 ul/test.
2. PI (KC-147): Use 5 ul test. Note: PI, a ready-to-use nucleic acid dye, is used for two-color (pSIVA-IANBD + PI) assays. Omit PI for one color pSIVA-IANBD assays.
3. 10X PBS (KC-148): Dilute to 1X prior to use.
4. 10X Binding Buffer (KC-149): Dilute to 1X prior to use.

### Before you begin

**Prepare 1X PBS:**

Dilute one part 10X PBS (KC-148) to 9 parts distilled H20 to make 1X PBS.

Determine the number of vials to be run.
Prepare 2 ml 1X PBS per vial.

**Prepare 1X Binding Buffer**

Dilute one part 10X Binding Buffer (KC-149) to 9 parts distilled H20 to make a 1X Binding Buffer.

Determine the number of vials to be run.
Prepare 0.5 ml 1X Binding Buffer per vial. Example: 10 vials = 5 ml 1X Binding Buffer

**Example: If you wish to run 10 vials, prepare 20 ml 1X PBS and 5 ml Binding Buffer**
**See Table III for an example of how to set up staining runs**

**Staining**

1. Pellet cells by spinning down tubes at 300 x g for 5 min. Carefully remove supernatant leaving the undisturbed pellet in the tube.
2. Wash cells twice with 1 ml of cold 1X PBS per tube After each wash, spin down at 300 x g for 5 min to pellet cells and carefully remove supernatant leaving behind the undisturbed pellet.
3. Resuspend cell pellet in 1X Binding Buffer at a concentration of $1 \times 10^6$ – $1 \times 10^7$ cells/ml. Take caution to resuspend gently by flicking tube, DO NOT VORTEX.

| Vial (tube) # | Cells | Stain | Notes |
|---|---|---|---|
| 1 | Untreated experimental cells | none | compensation set up |
| 2 | Untreated experimental cells | 5 ul pSIVA-IANBD | compensation set up |
| 3 | Untreated experimental cells | 5 ul PI | compensation set up |
| 4 | Untreated experimental cells | 5 ul pSIVA-IANBD + 5 ul PI | baseline cell death |
| 5 | *Treated experimental cells | 5 ul pSIVA-IANBD + 5 ul PI | treated profile |
| 6 | *Treated positive control cells | 5 ul pSIVA-IANBD + 5 ul PI | optional positive staining control |

**Table III. Tube set up: sample flow cytometry staining run.** *There are a myriad of protocols for inducing apoptosis/cell death. Researchers are encouraged to consult the scientific literature for additional information and protocols.

4. Transfer 100 ul of the resuspended cells (1 x $10^5$ – 1 x $10^6$ cells) to each vial that will be run.
5. Two color staining: Add 5 ul pSIVA-IANBD + 5 ul PI to each tube. Gently swirl to mix. Note: Omit PI for for one color pSIVA-IANBD staining.
6. Incubate for 20 min at RT (20-25°C) in the dark.
7. Add 400 ul of 1X Binding Buffer to each tube.
8. Analyze cells by flow cytometry immediately (Excitation = 488 nm, Emission = 530 nm) using the FL1 channel for detecting pSIVA-IANBD and the FL2 channel for detecting PI. Since apoptosis/cell death is a dynamic process, delays in analysis may obscure results which may change over time.

***Note:*** *pSIVA-IANBD and PI dilutions are provided as guidelines, researchers may need to optimize for their own model systems.*

**See Figs 4 and 5 for examples of one (pSIVA-IANBD) and two color (pSIVA-IANBD + PI) staining, respectively.**

## Suggested controls

### Compensation

The following controls are used to set up compensation and quadrants:

1. Unstained cells
2. Cells stained with pSIVA-IANBD only (no PI)
3. Cells stained with PI only (no pSIVA-IANBD)

### Positive control staining

A cell line, such as Jurkat or HL60, that can be readily induced to undergo apoptosis is recommended to obtain positive control staining. See Figs. 4 and 5 for examples using these cell lines, respectively.

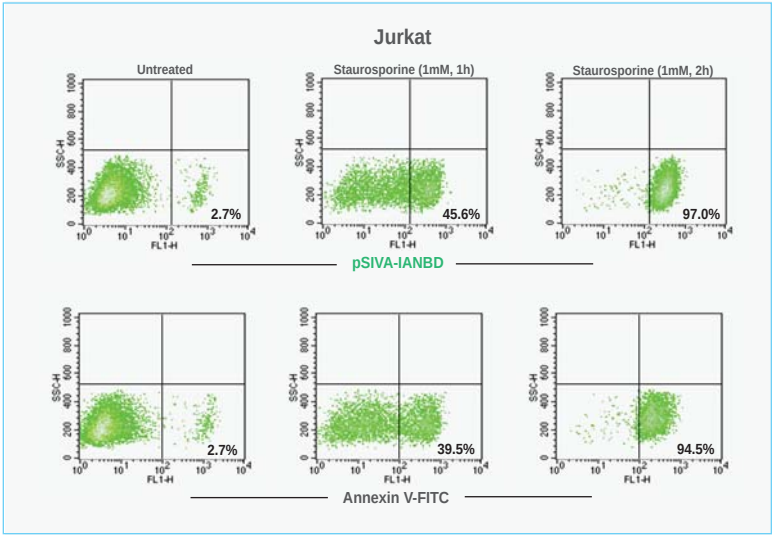


**Figure 4. Comparison of pSIVA-IANBD and Annexin V-FITC* in Flow Cytometry**
**The staining patterns were similar for the two dyes:**
1. Untreated: Primarily negative indicating healthy cells. The small positive population represents the basal level of cell death in the culture.
2. Staurosporine (1 h): Large positive population indicating active apoptosis. Staining in the 'negative' population gate has spread out, indicating movement towards cell death.
3. Staurosporine (3 h): Nearly the entire population is positive, indicating massive cell death.

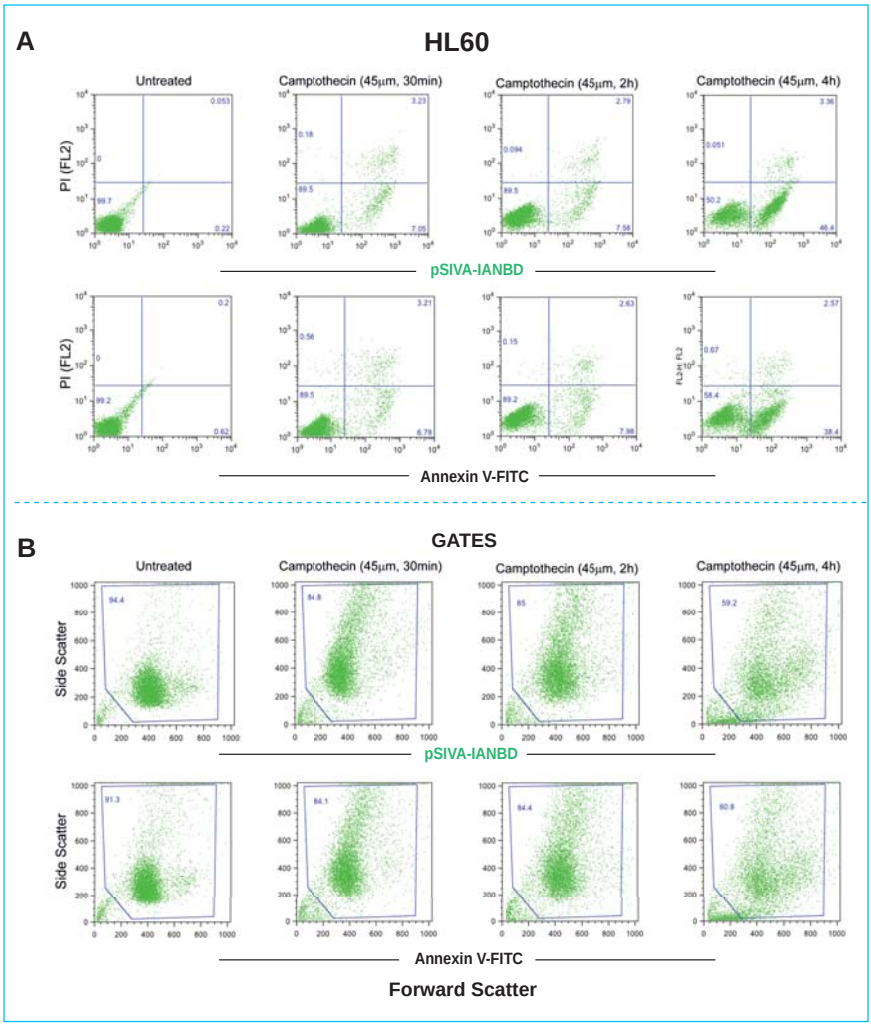


**Figure 5. Comparison of pSIVA-IANBD + PI and Annexin V-FITC* + PI in Flow Cytometry**
The staining patterns were similar for the two dyes. Evidence of camptothecin induced cell death was apparent after only 30 min as indicated by an increase in the single and double positive populations. The cell population FSC-H vs SSC-H scatter plots became more diffuse over time, another indication of treatment effects.

**Data Analysis**

The basal level of apoptosis and necrosis varies considerably within a given cell population. Even in the absence of induced apoptosis most cell populations will contain a minor percentage of cells that are positive for apoptosis (pSIVA-IANBD positive, PI negative) and dead, necrotic, or in the late stages of apoptosis (pSIVA-IANBD positive, PI positive). Thus an untreated cell population is used to define the basal level of apoptotic and dead cells.

Determine the percentage of cells that have been induced to undergo apoptosis by substracting the percentage of apoptotic cells in the untreated from the treated population. Since cell death is the eventual outcome of cells undergoing apoptosis, cells in the late stages of apoptosis will have a damaged membrane and stain positive for PI as well as for pSIVA-IANBD. Consequently cells that have undergone necrosis are not distinguishable from those which have undergone apoptosis.

It is important to note that a high level of basal apoptosis or cell death, in the absence of a cell treatment, may be indicative of an unhealthy cell culture. In these cases, researchers may need to optimize their cultures prior to treatment in order to properly assess the effects of treatment on cell death.

**Comparison of pSIVA-IANBD and Annexin V-FITC Staining**

Heretofore, Annexin V has been the standard reagent for detecting PS exposure. We have found that pSIVA-IANBD flow cytometry profiles are similar to those of Annexin V-FITC in standard apoptosis induction protocols (Figs. 4 and 5). Nuances as well as additional pSIVA flow cytometry applications remain to be fully elucidated.

**Annexin V-FITC is part of the CytoGLO™ Annexin V-FITC Apoptosis Detect Kit, Cat No. 10085K.*

# VIII. MICROSCOPY PROTOCOLS

## pSIVA™-IANBD Apoptosis/Viability Microscopy Kit

**Cat No:** IMG-6701K (Sufficient for 10 ml Staining Solution)

### Before you begin

Please note that pSIVA-IANBD binding to PS requires the presence of $Ca^{2+}$ , and that sufficient levels are usually present in most cell culture medium formulations (~1-2 mM $Ca^{2+}$) (Kim et al, 2010B). However if your medium lacks $Ca^{2+}$, it should be supplemented with 2 mM $Ca^{2+}$ prior to adding pSIVA-IANBD.

**Reagents**

1. pSIVA-IANBD (KC-150): Use 10-20 ul/ml.
2. PI (KC-147-500): Use 5-10 ul/ml. Note: PI, a ready-to-use nucleic acid dye, is used for two-color (pSIVA-IANBD + PI) assays. Omit PI for one color pSIVA-IANBD assays.

### Basic Fluorescence Microscopy

**Staining**

1. Add 10 -20 ul /ml pSIVA-IANBD + 5-10ul/ml PI directly to cultures. Omit PI for one color pSIVA-IANBD staining
2. Observe under the microscope. Use the green fluorescence filter set for pSIVA-IANBD (excitation maximum 488 nm and emission maximum 530 nm) and a red fluorescence filter set for PI (omit PI for one color staining)
3. Results may be seen immediately or within 5-10 min. Since apoptosis is a dynamic process, results may change over time.

***Note:*** *pSIVA-IANBD and PI dilutions are provided as guidelines, researchers may need to optimize for their own model systems.*

### Time-Lapse Live Cell Imaging: Introductory Remarks

pSIVA-IANBD is added directly to cell cultures to image real-time, chronological and dynamic events occurring during apoptosis (Fig 6):

1. Monitor progression of cell death.
2. Monitor cell-to-cell variations in response to treatments.
3. Assess differences in vulnerabilities of individual cells to death stimuli.

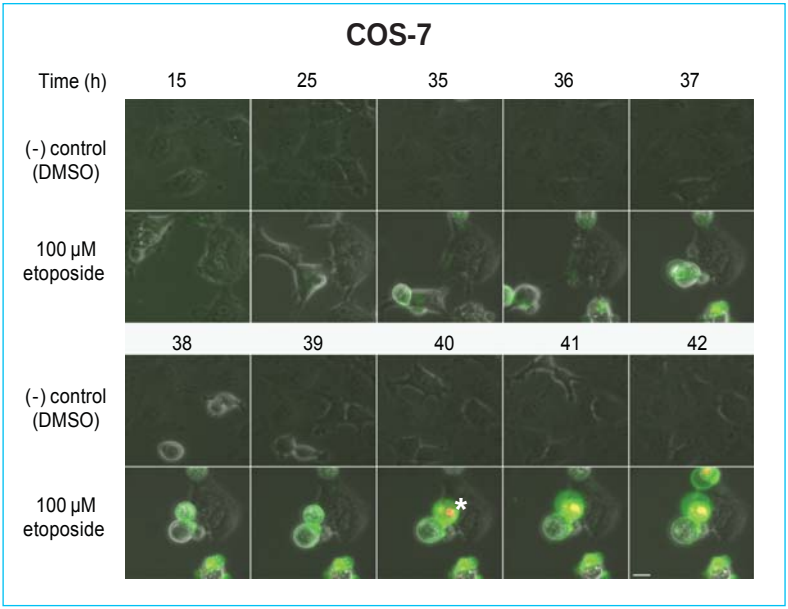


**Figure 6. Real-time live cell imaging of apoptosis:** COS-7 cells. COS-7 cells were induced to undergo apoptosis with etoposide (100 uM) or left untreated [DMSO (-) control]. pSIVA-IANBD + PI was added directly to the culture media and cells were imaged (37 degrees C, 5% CO2) by time lapse microscopy of the same fields over time. Merged channels of green and red fluorescence are shown. Green fluorescence indicates pSIVA-IANBD binding to PS exposed on the outer leaflet of the plasma membrane, and orange-yellow fluorescence indicates PI staining of nuclei. pSIVA-IANBD stained cells prior to PI staining. *Time point when PI staining was first seen in the cell; PI staining indicates loss of membrane integrity which is characteristic of late apoptotic or dead cells. Figure from Kim et al (2010a).

4. Identify where the first subcellular responses occur can be monitored.
5. Measure viability of cells without perturbing experimental conditions.

Researchers are encouraged to view pSIVA Assays in the live-cell, time-lapse imaging videos of DRG neurons during apoptosis and rescue (Kim et al, 2010a, Supplementary Videos 1-4).

A gradual increase in pSIVA-IANBD staining was seen in treated cells which was concurrent with progression towards cell death as indicated by PI staining during later time points. Little or no staining was seen in untreated cells.

## Time-lapse Imaging: Basic protocol

This protocol is adapted from Kim et al (2010b). The optimal protocol for your experiments may vary depending on your imaging equipment and experimental model system. A six-well plate is used as an example, other configurations may be also be used.

1. Turn on the fluorescence lamp, incubation system (37°C, 5% CO2 in a humidified atmosphere), motorized stage, microscope and camera. Let the system equilibrate at 37°C for 2-3 h. Also equilibrate the plate temperature in the microscopy incubation chamber ~ 1h before imaging. Equilibration is an essential step for time-lapse imaging experiments using an automated system with programmed positions and focus points because slight changes in temperature in the system can disrupt the focus.
2. Add 10-20 ul/ml pSIVA-IANBD and 5-10 ul/ml to cultures. Use one well as a negative control (no reagents).
3. Set up the imaging program with the microscope software as follows: time course of imaging and time interval for capturing images.
4. Set fluorescence channels and exposure times. For pSIVA use the green fluorescence filter set (excitation maximum 488 nm and emission maximum 530 nm). Minimize bleaching during extended time-lapse imaging experiments by decreasing the exposure time and/or incorporating an EMCCD (electron multiplying charged coupled device) camera for the time-lapse experiments (for example, Photometrics Cascade 1K). Note: Particular care should be taken for experiments aimed at imaging the reversal of pSIVA-IABND binding, which is indicative of transient PS exposure, rescue from apoptosis/cell death or rescue from neuronal degeneration.
5. Pick the positions and focal depth for the different fields of view to be imaged.
6. Recheck the position and focus for each field of view before starting the time-lapse imaging experiment.
7. Analyze results at the end of experiment.

## Analysis of Viability

pSIVA can be used as a non-invasive tool to measure cell viability during experiments, without disrupting experimental conditions.

Methodology: Add pSIVA-IANBD to cell cultures at any time during experiments and monitor fluorescence of individual cells. For example, Figure 6 shows that untreated cells (DMSO control) remained viable (pSIVA-IANBD negative, PI negative) during a 42 h monitoring period whereas treated cells (100 uM etoposide) became progressively positive for pSIVA-IANBD and PI.

## Detection of Transient PS Exposure

Although PS exposure is most well known as an irreversible event that occurs during apoptosis, the phenomenon of transient exposure in healthy cells is increasingly being recognized (Fig. 7). For example, transient PS exposure occurs during lymphocyte activation, vesicle release, muscle cell differentiation, embryonic development and other events that are accompanied by changes in plasma membrane structure (Reviewed in Green, 2010; Bevers and Williamson, 2010b; Kim et al, 2010b).

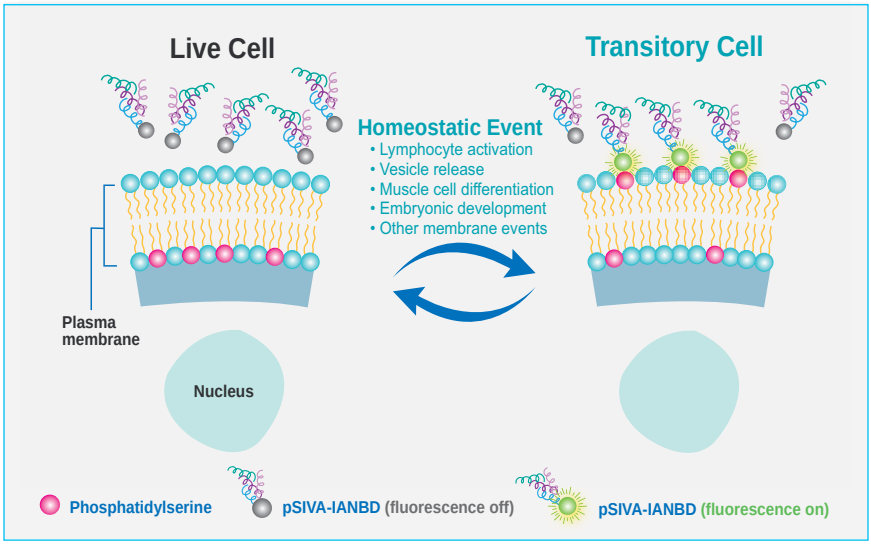


**Figure 7. Ephemeral or fleeting pSIVA-IANBD fluorescence signals accompany transient PS exposures associated with normal physiological or homeostatic events (artist's rendition).** When PS flips back to the inner membrane following transient PS exposure, pSIVA-IANBD will be released back into the medium and fluorescence lost. Potential areas of study are shown in the cartoon. Note: The phenomenon of transient pSIVA-IANBD fluorescence exposure has been observed in unperturbed cultures as rapid on/off fluorescence, and is thought to represent normal membrane event. However, this is a wide open area of study and details remain to be elucidated.

However, heretofore there is a paucity of research tools to fully analyze transient exposure of PS and the mechanisms remain elusive. The advent of pSIVA-IANBD, with its polar-sensitive binding properties now enables researchers to investigate this fascinating phenomenon (Kim et al, 2010a/b). pSIVA-IANBD has already shown that transient or reversible PS exposure may also occur when a cell death program is initiated but aborted or rescued prior to a point of 'no return' (Fig. 8 and Kim et al, 2010a/b).

Methodology: Transient PS exposure has been studied by live imaging. Transient versus apoptotic PS events can be distinguished by setting the time frame of imaging to match the time frame of the process (Kim et al, 2010b). For example, transient PS exposure during vesicle release in healthy cells may occur on a time scale of seconds to minutes before PS is restored to the inner leaflet. In contrast, PS remains exposed on the outer leaflet for hours to days during apoptosis, either until cell death or rescue occurs. As the phenomenon of transient PS exposure is an emerging area of study, researchers will need to empirically determine the optimal imaging time frames for their model system and the process they are evaluating.

## Analysis of Degeneration and Rescue: Primary Neurons

DRG neurons are useful model systems for survival and degeneration because they are only dependent on one growth factor for survival, NGF, which can easily be removed. Removal of NGF leads to degeneration. Axonal degeneration involves apoptotic mechanisms which do not always result in cell death (reviewed in Saxena and Caroni, 2007). Hence, neuronal degeneration/rescue studies can be used to help define critical periods or windows when apoptosis processes are still reversible (reviewed in Kim et al, 2010a/b).

The protocols are adapted from Kim et al, 2010a/b.
The experiments were done with six-well plates* of purified primary dorsal root ganglion (DRG) that were still dependent on NGF for survival (cultured 1-2 weeks). The DRG neurons were cultured in etched wells in order to orient growth along a single axis. Note: For NGF deprivation experiments, neurons should be used after 1-2 weeks because after about 3 weeks the neurons will be independent of NGF for survival.*Experiments should ideally be performed on tissue culture treated plastic. If using glass coverslips, they must be attached to culture wells before plating cells to prevent coverslips from shifting during imaging. Coverslips can be attached to wells with nontoxic adhesives (slicon caulk, cyanoacrylate) or with a drying substrate such as collagen.

### Neuronal Degeneration Protocol

1. Begin with Step 1 of the “Time-lapse imaging: Basic protocol” to equilibrate microscopy system.
2. Remove NGF from the culture media to initiate degeneration in specific wells. Wash cells three times with medium without NGF. Other suitable protocols may also be used to induce degeneration. For a negative control (no induction of degeneration), replace the NGF back into one well.

3. Follow Steps 2-7 of the “Time-lapse imaging: Basic protocol. Note: An imaging interval of 30 m is suggested, neurons will degenerate over 24-40 h.

Anticipated results: PS exposure on the axon can be detected by pSIVA-IANBD as early as 10 h in 1 week old DRG neurons that have been induced to undergo degeneration by removal of NGF (Kim et al, 2010b). PS exposure has been found to start first in localized areas of the axon and then spread progressively in either direction, towards the cell body or axon (Figs. 8 and 9). pSIVA-IANBD and PI staining in the cell body occurred later (Fig. 10). There will likely be cell-cell variability and heterogeneity in the initiation and progression of PS exposure, and thus pSIVA-IANBD fluorescence patterns in the degenerating neurons.

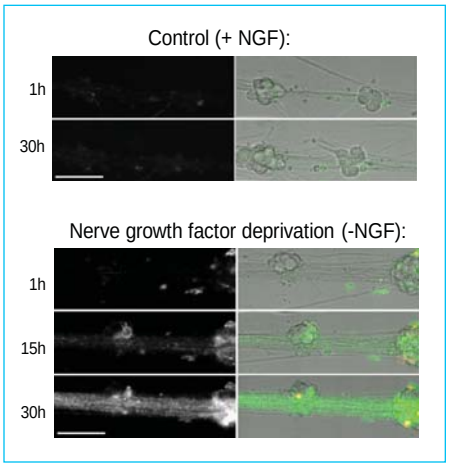


**Figure 8. Monitoring the degeneration of neurons with pSIVA-IANBD.** Time-lapse microscopy of rat DRG neurons with NGF (normal physiological conditions) (a) and under NGF deprivation (b). pSIVA-IANBD + PI were present in the culture medium for the duration of the experiment. pSIVA-IANBD fluorescence (left panels) and merged images of phase contrast, green (pSIVA-IANBD) and red (PI) fluorescence (right panels) are shown. Increasing fluorescence was seen -NGF but not +NGF cultures over time, indicating neuronal degeneration in the -NGF but not +NGF cultures. 100 um scale bar. Figure from Kim et al (2010a).

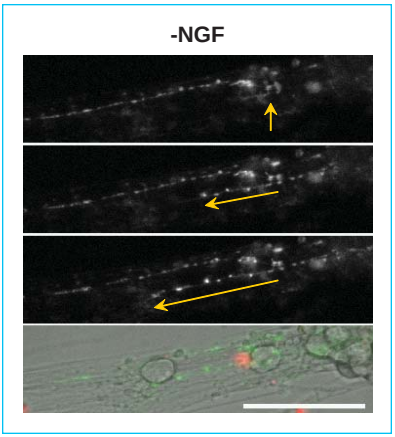


**Figure 9. Spatiotemporal analysis of membrane changes along a single degenerating axon with pSIVA-IANBD.** Time-lapse images at 20 min intervals of a rat DRG axon from a -NGF culture showing progression of the pSIVA-IANBD punctate staining of PS exposure. Black and white: pSIVA-IANBD fluorescence. Bottom panel: and merged images of phase contrast, green (pSIVA-IANBD) and red (PI) fluorescence. 100 um scale bar. Cells were imaged with pSIVA-IANBD and PI present in the culture medium for the duration of the experiment. Figure from Kim et al (2010a).

**Neuronal Rescue Protocol**

1. Neuronal degeneration is initiated as described in Step 2 of the Neuronal Degeneration Protocol.
2. Add NGF back to cultures once PS exposure (pSIVA-IANBD fluorescence) is detected on the axons. Different time points should be used to determine the window of rescuability in your model system. For example, Kim et al (2010a) added NGF back to cultures at 7, 10 and 15 h after initial NGF removal (Fig. 11).
3. Quantify the recovery from degeneration by measuring the total fluorescence of different fields of view at low magnification in both control (-NGF) and rescued (-NGF followed by +NGF) cultures. Increasing pSIVA-IANBD fluorescence indicates increasing PS exposure. Decreasing fluorescence in rescued cultures is considered to indicate reversal of PS exposure.

Expected results: Adding NGF back to the cultures will result in a reduction of fluorescence in neurons that have been rescued (Figs. 11 and 12). Neurons already showing signs of apoptosis (pSIVA-IANBD) will vary in their ability to be rescued. There appears to be a critical window for rescuability with respect to pSIVA-IANBD fluorescence (Kim et al 2010a/b). pSIVA-IANBD positive staining typically begins in the axon and moves towards the cell body. Cells appear to be lose their ability to be rescued once pSIVA-IANBD staining is seen in the cell body (Fig. 13).

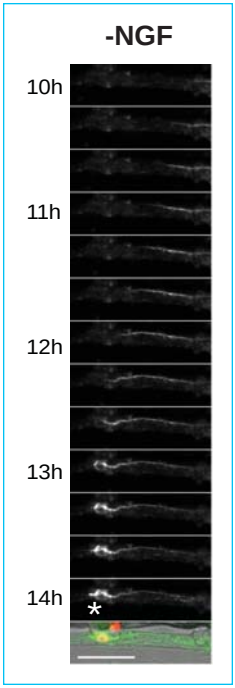


**Figure 10. Time-lapse images showing the progressive movement of PS exposure along axons to the cell body as detected by pSIVA-IANBD.** Images were taken 10-14 h (3 frames/h) after NGF removal. *Indicates where PI staining was first seen in the cell body. Black and white: pSIVA-IANBD fluorescence. Bottom panel: Merged images of phase contrast, green (pSIVA-IANBD) and red (PI) fluorescence. 100 um scale bar. Cells were imaged with pSIVA-IANBD and PI present in the culture medium for the duration of the experiment. Figure from Kim et al 2010a.

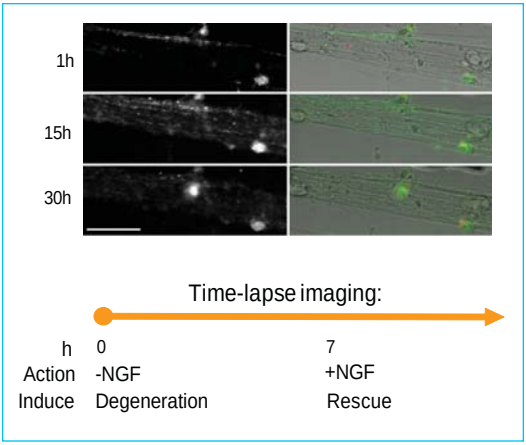


**Figure 11. Rescue of axonal degeneration.** DRG neurons that were dependent on NGF were induced to undergo cell death by NGF deprivation for 7 h before re-addition of NGF to the culture media. Note the reduction in fluorescence between 15 h and 30 h, indicative of a reduction of pSIVA-IANBD binding. See Fig. 12 for a quantitative analysis of rescued versus non rescued cultures. Black and white: pSIVA-IANBD fluorescence. Color: Merged images of phase contrast, green (pSIVA-IANBD) and red (PI) fluorescence. 100 um scale bar. Cells were imaged with pSIVA-IANBD and PI present in the culture medium for the duration of the experiment. Figure from Kim et al 2010a.

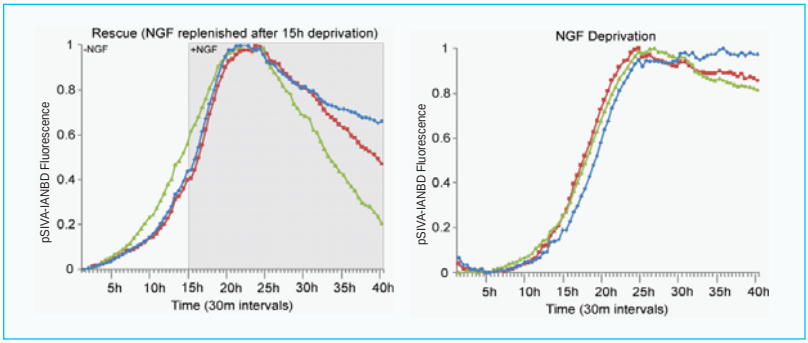


**Figure 12. Quantitative analysis of pSIVA-IANBD fluorescence in rescued and NGF deprived neuronal cultures.** Rescue of axonal degeneration. DRG neurons that were dependent on NGF were induced to undergo cell death by NGF deprivation 15 h before re-addition of NGF to the culture media. Total fluorescence was measured for different fields at low magnification. pSIVA-IANBD fluorescence decreased in cultures where NGF was re-added compared to cultures which were deprived of NGF throughout the entire 40 h monitoring period. Variations in the amount of rescue (decreasing total fluorescence) reflects heterogeneity in the ability of individual neurons to be rescued as well as differences in the time frame needed for rescue. Cells were imaged with pSIVA-IANBD and PI present in the culture medium for the duration of the experiment. Figure from Kim et al 2010a.

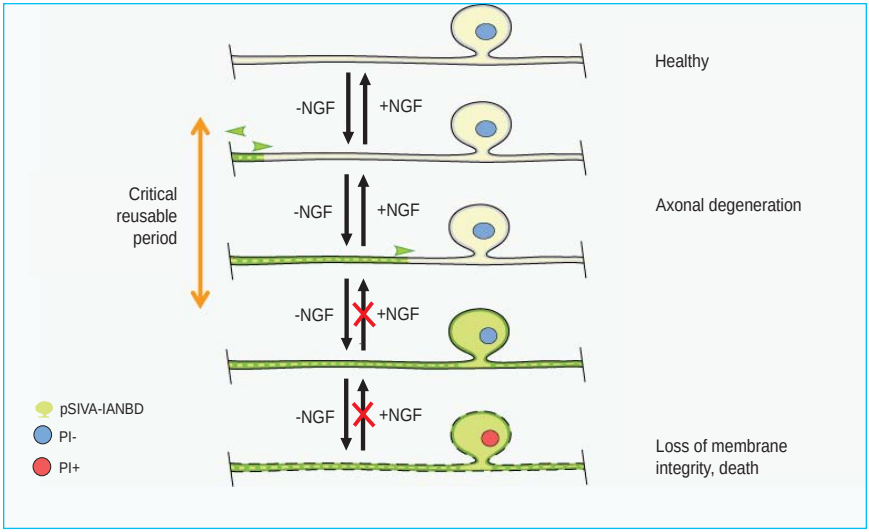


**Figure 13. Critical window for neuronal cell rescue.** pSIVA-IANBD staining indicates that PS exposure during apoptosis starts on the axons and initially may move towards or away from the cell body. Eventually the cell body becomes positive, followed by PI staining indicating loss of membrane integrity and impending death. Rescue appears to be possible only before PS exposure/pSIVA-IANBD fluorescence has progressed to the cell body. Green: pSIVA-IANBD fluorescence. Red: PI fluorescence.

## *In Vivo* and Tissue Applications

pSIVA-IANBD can be injected into animals or added directly to fresh tissue (Kim et al, 2010). In Fig 14, pSIVA-IANBD was injected intramuscularly along a rat sciatic nerve 3 days after nerve transaction and degenerating neurons were imaged. Except for some minor staining of tissues damaged while exposing the nerve for imaging, pSIVA-IANBD exclusively stained axons on the sciatic nerve distal to the site of the injury (Fig 14). Punctate staining was observed, similar to that seen in the degenerating axons of DRG neurons *in vitro*. In contrast, staining in the contralateral control (uninjured) nerve was undetectable (Fig 15). It is noteworthy that when Annexin V-FITC was added to the uninjured nerve, the background signal was significant thereby underscoring the difficulty in using Annexin V-FITC for live imaging experiments.

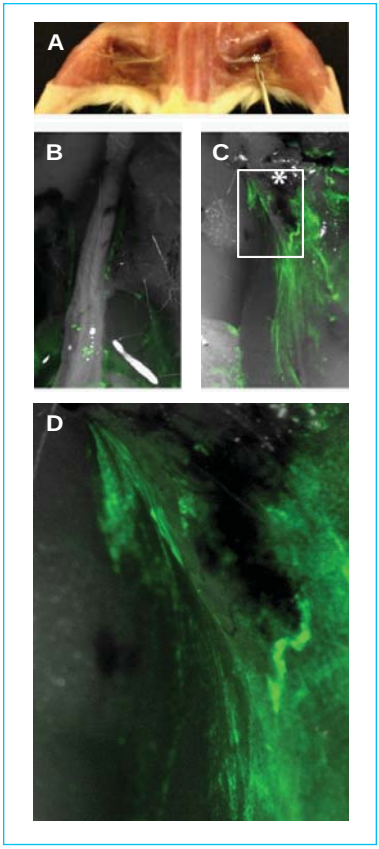


**Figure 14. Imaging of axonal degeneration with pSIVA-IANBD.** A. Exposed sciatic nerves of a rat prior to transection on the right side. B, C. Overlay of the pSIVA-IANBD fluorescence and brightfield images of the untransected (N) and transected (V) sciatic nerve. pSIVA-IANBD was injected intramuscularly, along the sciatic nerve before imaging. *The nerve was transected just below the greater trocanter as denoted by the asterisks in A and C. D. High magnification of C, showing the pattern of pSIVA-IANBD fluorescence staining along axons in the injured nerve. Figure from Kim et al 2010a.

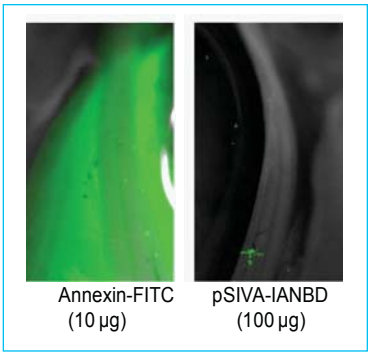


**Figure 15. pSIVA-IANBD lacks background fluorescence compared to Annexin V-FITC.** Overlay of fluorescence and brightfield images of an uninjured sciatic nerve stained with Annexin V-FITC or pSIVA-IANBD. Even at a 10 fold greater application, pSIVA-IANBD (100 ug) lacks background fluorescence compared to Annexin V-FITC (10 ug). The low background of pSIVA-IANBD makes it particularly suitable for live-cell imaging compared to Annexin V-FITC. Figure from Kim et al 2010a.

## IX. FREQUENTLY ASKED QUESTIONS

1. **Is pSIVA-IANBD in the cell culture harmful to cells?** We do not have any evidence that pSIVA-IANBD is harmful to cells or perturbs the cell culture. Furthermore, no differences in cell growth rate were observed when COS-7 cells were incubated in the presence or absence of pSIVA-IANBD (Kim et al, 2010a).
2. **Does unbound pSIVA-IANBD in solution have background fluorescence?** pSIVA-IANBD fluorescence in the solution (unbound) state is negligible in comparison with the membrane-bound state (~50 fold increase) (Kim et al, 2010B).

3. **If pSIVA-IANBD is inadvertently used in excess, will background increase?** Since unbound pSIVA-IANBD lacks fluorescence, background will not appreciably increase in the presence of moderate excess of pSIVA-IANBD.
4. **Can pSIVA-IANBD be used for high-throughput screening?** Yes since pSIVA-IANBD fluorescence correlates directly with binding to apoptotic cell membranes, pSIVA-IANBD fluorescence can be quantified using a multiplate reader for high-throughput screening of conditions that cause or prevent cell death.
5. **Is pSIVA-IANBD compatible with fixation?** Yes, please see Zhang et al (2011, Fig. 4) for an example of pSIVA-IANBD staining of live cells and then fixation prior to analysis.

## X. TROUBLESHOOTING GUIDE

| Problem | Possible Reasons | Solution |
|---|---|---|
| No pSIVA-IANBD fluorescence | $Ca^{2+}$ is not present in the culture media | Check medium formulations and make sure that the $Ca^{2+}$ ($CaCl_2$) concentration is ~1-2 mM |
| | Suboptimal microscopy settings | Make sure that the live-imaging setup has been equilibrated at 37°C, that the microscope settings are correct for the camera, fluorescence and exposure times, and that the focus positions are correct. |
| | PS exposure is not present | Use a positive control cell line that is readily induced to undergo apoptosis to validate the system |

## XI. REPORTING RESULTS IN THE SCIENTIFIC LITERATURE

Historically, researchers have used various expressions such as 'percent apoptosis', 'percent cell death', 'percent necrosis' and 'percent cell survival' to report results in the literature. However, these expressions can be confusing and imprecise, and in 2005 the Nomenclature Committee on Cell Death (NCCD) recommended abandoning them (Kroemer et al, 2005). Since then the NCCD has urged authors to replace nebulous terms with more precise terms that reflect the methodology, and also periodically publishes updated guidelines (Kroemer et al, 2009). The suggestions of the highly regarded NCCD are increasingly being accepted and adopted in the literature (Google Scholar: www/scholar.goggle.com : search words 'NCCD', 'cell', 'death' to identify representative publications).

For example, the results of the pSIVA Assay would be reported as 'percent pSIVA-binding' rather than 'percent apoptosis'. Other descriptive terms, depending on the biological phenomena assayed, might include:

'percent PI positive', 'percent Annexin V positive', 'percent active caspase-3 positive', 'percent cells with a low mitochondrial potential' and 'percent TUNEL positive cells'. This list of recommended terminology is not exhaustive, but rather intended to help standardize the literature. For example, the term 'percent apoptosis' may mean different things to different researchers compared to 'percent pSIVA-IANBD' binding which clearly denotes what is measured. IMGENEX strongly encourages investigators to consider these recommendations when publishing their results.

## XII. GENERAL REFERENCES

Bevers EM, PL Williamson. Phospholipid scramblase: An update. *FEBS Letters.* 584:2724-2730 (2010). **The mechanism of apoptosis/cell death induced PS externalization remains to be fully elucidated; this review describes the state of knowledge.**

Hammill AK, JW Uhr, RH Scheuermann. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. *Experimental Cell Res* 251:16-21 (1999). **An early demonstration that PS exposure during apoptosis may be reversible. However, this concept was not developed and PS exposure gained widespread recognition as an**

**"irreversible" hallmark event occurring early during apoptosis. The advent of pSIVA-IANBD offers researchers a novel tool to explore the concept of transient PS exposure.**

Kroemer G, L Galluzzi, P Vandenabeele, J Abrams, ES Alnemri, EH Baechrecke, MV Blagoskionny, WS El-Deiry, P Golstein, DR Green, M Hengartner, RA Knight, S Kumar, SA Lipton, W Malorni, G Nunez, ME Peter, J Tschopp, J Yuan, M PIacentini, B Zhivotovsky, G Melino. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differentiation* 16:3-11 (2009). **Updated NCCD guidelines for reporting cell death assay results.**

Kroemer G, WS El-Deiry, P Golstein, ME Peter, D Vaux, P Vandenabeele, B Zhivotovsky, MV Blagosklonny, W Malomi, RA Kinght, M Piacentini, S Nagata, G Melino. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differentiation* 12:1463-1467 (2005). **Revamping the way investigators report cell death results, from cell death "type" to mechanism based.**

Martin SJ, CPM Reutelingsperger, AJ McGahon, JA Rader, RCAA van Shie, DM LaFace, DR Green. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpession of Bcl-2 and Abl. *J Exp Med* 182:1545-1556 (1995). **A landmark, early paper describing the use of Annexin V to detect apoptosis.**

Saxena S, P Caroni. Mechanism of axon degeneration: From development to disease. *Prog Neurobiol* 83:174-191 (2007). **A perspective on the phenomenon of axon degeneration which resembles apoptosis but also involves cell survival.**

Vermes I, C Haanen, H Steffens-Nakken, C Reutelingsperger. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 184:39-51 (1995). **Discovery and development of the acclaimed Annexin V apoptosis assay.**

## XIII. pSIVA™ PRODUCT CITATIONS


1. Kim YE, J Chen, JR Chan, R Langen. Engineering a polarity-sensitive biosensor for time-lapse imaging of apoptotic processes and degeneration. *Nat Methods* 7:67-73 (2010a). **Real-time live-cell imaging and time-lapse microscopy of apoptosis: Fig 2 (COS-7 cells), Fig 3 (rat neuronal degeneration), Fig 4 (rat axonal degeneration), Fig 5 (rescue of rat neuronal degeneration as visualized by pSIVA).**

2. Kim YE, J Chen, R Langen, JR Chan. Monitoring apoptosis and neuronal degeneration by real-time detection of phosphatidylserine externalization using a polarity-sensitive indicator of viability and apoptosis. *Nature Protocols* 5:1396-1405 (2010b). **Time lapse microscopy of neurons in normal survival conditions and after NGF deprivation (Fig 2).**

3. Zhang CQ, Yeh T-I, A Leyva, LG Frank, J Miller, YE Kim, R Langen, S Finkbeiner, ML Amzel, CA Ross, MA Poirier. A compact B model of huntingtin toxicity. *JBC* 286:8188-8196 (2011). **A pSIVA-IANBD based cell suspension toxicity assay was used to determine cell viability in mouse Neuro2A (neuroblastoma) overexpressing huntingtin proteins (Fig 4).**

4. Krajewska M, Z You, J Rong, C Kress, X Huang, J Yang, T Kyoda, R Leyva, S Banares, Y Hu, C-H Sze, MJ Whalen, L Salemena, R Hakem, BP Head, JC Reed, S Krajewski. Neuronal deletion of caspase 8 protect against brain injury in mouse models of controlled cortical impact and kainic acid-induced excitotoxicity. *PLOS ONE* 6(9): e24341. doi:10.1371/journal.pone.0024341 (2011). **IF (mouse primary neuron cultures): Figs 2B, 3. Cells were labeled with pSIVA-IANBD for 15 min, fixed and stained with a MAP2 antibody and DAPI. Cells double-stained with both pSIVA-IANBD and MAP2 (neuronal marker) were identified as degenerating neurons.**

5. Graewe S, KE Rankin, C Lehmann, C Deschermeier, L Hecht, U Froehlke, RR Stanway, V Heussler. Hostile takeover by Plasmodium: reorganization of parasite and host cell membranes during liver stage egress. *PLOS ONE* 7(9): e1002224 doi:10.1371/journal.ppat.1002224 (2011). **IF (HepG2 cells infected with P. berghei parasites), Fig 1**


***Note:*** *Figs.3, 6-15 in the manual are from Kim et al (2010a/b).*

## NOTES