

Monitoring apoptosis and neuronal degeneration by real-time detection of phosphatidylserine externalization using a polarity-sensitive indicator of viability and apoptosis

Yujin E Kim¹, Jeannie Chen², Ralf Langen¹ & Jonah R Chan¹

¹Department of Biochemistry and Molecular Biology, Zilkha Neurogenetic Institute at the Keck School of Medicine, University of Southern California, Los Angeles, California, USA. ²Department of Cell and Neurobiology, Zilkha Neurogenetic Institute at the Keck School of Medicine, University of Southern California, Los Angeles, California, USA. Correspondence should be addressed to J.R.C. (jonah.chan@usc.edu) and R.L. (langen@usc.edu).

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Applications for noninvasive real-time imaging of apoptosis and neuronal degeneration are hindered by technical limitations in imaging strategies and by existing probes. Monitoring the progression of a cell through apoptosis could provide valuable insight into the temporal events that initiate cell death as well as the potential for rescue of apoptotic cells. We engineered an annexin-based biosensor to function as a polarity-sensitive indicator for viability and apoptosis (known as pSIVA) by binding to externalized phosphatidylserine (PS) exposed on apoptotic cell membranes. Constructed from a structure-based design strategy, pSIVA fluoresces only when bound to PS and remains effectively undetectable in solution. In this paper, we describe protocols for the design, expression, purification and labeling of pSIVA as well as for its application in time-lapse imaging of degenerating neurons in culture; the entire protocol can be completed in 2 weeks. The primary advantage of this method is the flexibility to use pSIVA, in combination with other probes and without perturbing experimental conditions, to explore the cellular mechanisms involved in apoptosis and degeneration in real time.

INTRODUCTION

Apoptotic processes are dynamic events that have central roles in various biological processes, ranging from the maintenance of homeostasis to the progression of pathological conditions. Although several biochemical assays aimed at detecting the different stages of the apoptotic pathway are available^{1–5}, there are few methods that allow continuous monitoring of the entire process. We recently developed a novel polarity-sensitive annexin-based biosensor that facilitates real-time imaging of apoptotic cell membrane changes⁶.

Annexin proteins are commonly used in apoptosis kits on the basis of their property of Ca²⁺-dependent binding to negatively charged lipids such as phosphatidylserine (PS)^{7,8}, which become exposed on apoptotic cell membranes⁴. Of the many changes that occur during apoptosis, externalization of PS on the surface of apoptotic cell membranes has been established as an early event in the pathway, common to various cell types and apoptotic conditions⁹. Although normally restricted to the inner leaflet of the plasma membrane¹⁰, after initiation of apoptotic processes PS is translocated to the external leaflet^{1,4,9}, providing easily accessible binding targets for continuous monitoring by time-lapse imaging techniques. Previously reported annexin-based protocols for detecting apoptosis in cultured cells typically involve incubating cells with fluorescently labeled annexin proteins, followed by dissociating the cells for analysis by flow cytometry or washing and fixing cells for analysis by fluorescence microscopy^{11,12}. To optimize the assay for detection and continuous imaging of apoptotic cells in real time, we modified annexin B12 with polarity-sensitive fluorophores to engineer a biosensor that emits high fluorescence intensity only when bound to PS-containing membranes. This newly developed polarity-sensitive indicator of viability and apoptosis is termed a pSIVA.

Design of pSIVA and development of the protocol

We designed pSIVA on the basis of previous structural studies of the annexin B12 protein^{13–17}. Annexins are a family of proteins that are defined by their structurally conserved core domains and their ability to bind reversibly and Ca²⁺-dependently to negatively charged membranes with high specificity to PS. Detailed structural studies of annexin B12 in the Ca²⁺-dependent membrane-bound state revealed that residues located in the loop regions on the convex side of the protein interacted directly with the hydrophobic environment of the lipid bilayer^{13,15,16}, transitioning from a polar environment in the solution state to a nonpolar environment in the Ca²⁺-dependent membrane-bound state. On the basis of their central position in membrane-binding loops, we engineered cysteine mutations at residues 101 and 260 in membrane-binding loops (Fig. 1) and labeled the cysteines with polarity-sensitive fluorophores. Fluorophores were selected on the basis of their property of emitting low fluorescence intensity in polar environments (for example, in solution) and emitting high fluorescence intensity in nonpolar environments (for instance, in membranes). Attaching polarity-sensitive fluorophores at amino acid positions 101 and 260 in annexin B12 confers a built-in switch for fluorescence emission, which is 'off' in the solution state and turns 'on' in the membrane-bound state⁶ (Fig. 1). The pSIVA design is also applicable to different types of annexins; using the same structure-based design strategy, annexin A5 can also be used to achieve similar results⁶, and should be labeled at positions 103 and 262.

Applications and advantages

Using the pSIVA method in combination with live-cell imaging techniques provides a means to track the progression and timing of a cell through apoptosis starting from the initial stages of the pathway.

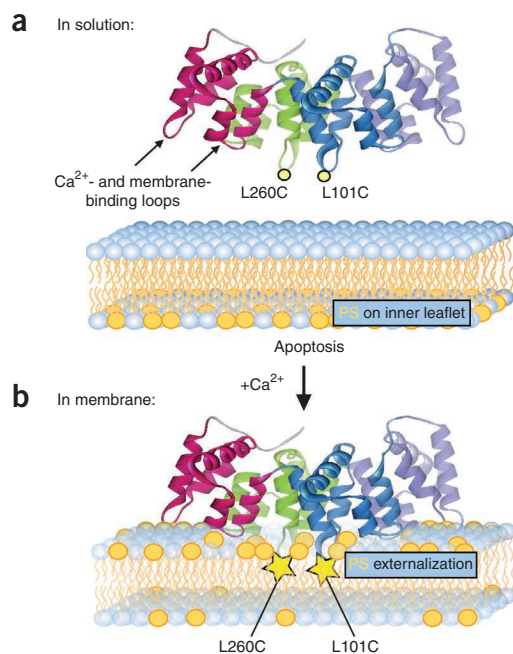


Figure 1 | Structure-based design of pSIVA. (a) pSIVA is represented using the crystal structure of annexin B12 (Protein Data Bank entry 1AEI)¹⁷, with the sites of the introduced cysteine mutations (L101C, L260C) and subsequent attachment of the IANBD labels highlighted with yellow spheres. Typically, viable cells restrict phosphatidylserine (orange lipid head groups) on the inner leaflet of the plasma membrane. Therefore, pSIVA remains in solution and the polarity-sensitive fluorophores do not emit any significant fluorescence (yellow circles). (b) Upon initiation of apoptotic processes and degeneration, phosphatidylserine translocates to the outer leaflet of the plasma membrane, whereby pSIVA selectively binds to the acidic phospholipid. On binding, the strategically placed IANBD labels are exposed to the nonpolar lipid environment, which results in a 'switching on' of fluorescence signal (yellow stars).

Other methods using conventionally fluorescently labeled annexins with fixed cells for fluorescence microscopy or dissociated cells for flow cytometry analysis are limited to observations at a single time point for a population of cells^{11,12}. A pSIVA is a polarity-sensitive mutant version of annexin B12, which is comparable to conventional fluorescently labeled annexins, but with added benefits: low signal-to-noise ratio, elimination of the need for washing, and continuous live-imaging capacity. Viable alternatives for monitoring apoptosis by live-cell imaging are not widely available, specifically because few probes allow detection and resolution at the single-cell level without perturbing the experimental conditions. Alternative methods suitable for live-cell imaging of cell death include using vital dyes such as propidium iodide, which stains the nuclei of necrotic cells or cells that have lost membrane integrity at late-stage cell death⁴; monitoring changes in cell morphology that are attributed to the different stages of the apoptotic pathway¹⁸; or using fluorochrome-labeled inhibitors of apoptosis (FLICAs) that detect the activation of specific caspases during the apoptotic pathway¹⁹. Although FLICAs have been shown to be relatively nontoxic to the cell, the FLICA assay requires uptake into the cell and the subsequent irreversible binding of the FLICA probe to the activated caspase. In addition, the binding of FLICAs to the activated caspases has been shown to promote cell survival. Thus, FLICA probes are unsuitable for experiments aimed at testing conditions that lead to rescue from degeneration or events occurring at the end stages of cell death.

The application of the pSIVA biosensor to imaging degeneration in neurons is particularly useful for visualizing the spatiotemporal progression of apoptotic membrane changes along the axon and cell body. As the binding target (PS) is on the plasma membrane, detection of apoptotic changes is not limited to a particular cell compartment, unlike assays for DNA fragmentation (terminal deoxynucleotidyl transferase dUTP nick end labeling assay) that limit detection to the nucleus or to activated caspases that can be localized specifically to either the cell body (caspase 3) or axon (caspase 6)^{20,21}. Moreover, the externalization of PS can be detected on initiation of apoptotic processes but before commitment to cell death, making it useful for investigating processes that could potentially block or induce rescue of degeneration⁶. Combined with other cellular markers, pSIVA can be used to investigate the underlying signaling pathways involved in initiating apoptotic processes and the progression of the apoptotic cascade. Furthermore, in combination with other probes, pSIVA can be used to detect different modes of cell death and in different time courses. For example, pSIVA and propidium iodide can be used to distinguish between apoptotic cells (stained initially by pSIVA, and then by propidium iodide at late-stage apoptosis) and necrotic cells (stained by both pSIVA and propidium iodide at the same time). In addition, as pSIVA binding is reversible, rescue from apoptotic processes can be observed by loss of pSIVA fluorescence, which is concurrent with loss of binding as PS is restored to the inner leaflet of the plasma membrane⁶.

Because pSIVA fluorescence in the solution state is negligible in comparison with the membrane-bound state (~50-fold increase), it can be used in excess without considering increases in background fluorescence⁶ or the need for titration to optimize the working concentration. In addition, as fluorescence correlates directly with binding to apoptotic cell membranes, quantification of pSIVA fluorescence can be used for high-throughput screening of conditions that cause or prevent cell death using a multiwell plate reader. Furthermore, no adverse effects have been observed in various cell lines and primary neuron cultures with the continuous presence of pSIVA in the medium (10 $\mu\text{g ml}^{-1}$)⁶.

The pSIVA method is also suitable for *in vivo* imaging applications. We previously demonstrated the use of pSIVA to specifically visualize individual degenerating axons in the rat sciatic nerve after injury, without the interference of background signal⁶. Although annexin A5 labeled with conventional fluorophores or infrared probes has been used for *in vivo* imaging of apoptosis in the retina^{22,23} and in the heart²⁴, protocols for its use require additional optimization of the image contrast and the signal-to-noise ratio between bound and unbound annexin probes. Previous protocols have also applied radiolabeled annexin for noninvasive, *in vivo* detection of apoptotic cells in tumors by positron emission tomography²⁵; however, this approach results in considerable background signal from the unbound protein in circulation and non-specific radiolabeling of organs such as the liver, spleen and kidney. Moreover, unlike fluorescence microscopy, imaging at single-cell resolution is not possible with positron emission tomography.

Additional considerations and limitations

Detection and binding of pSIVA to PS exposed on the surface of apoptotic cells require the presence of Ca^{2+} , which is generally fulfilled by most cell culture medium formulations (~1–2 mM Ca^{2+}). Although externalization of PS is predominantly a hallmark of apoptotic processes, transient PS exposures may occur during

other biological processes such as vesicle release or other events that are accompanied by changes in plasma membrane structure. However, these transient PS exposures can be easily distinguished by setting the time frame of imaging to match the time frame of the process. For example, transient exposure of PS during vesicle release in healthy cells may occur on the time scale of seconds to minutes, before PS is restored to the inner leaflet. During apoptosis and/or degeneration, PS remains exposed on the outer leaflet for hours to days, until cell death or rescue occurs.

An additional consideration is the possibility that pSIVA is internalized by the apoptotic cell (after binding to exposed PS) by a mechanism similar to that described for annexin A5 (ref. 26). Previous studies with labeled annexin A5 (ref. 26) and our previous study with pSIVA⁶ have shown that the possible internalization of pSIVA does not affect the ability of cells to undergo cell death or recover from apoptotic processes. However, it is possible to experience a loss of signal as pSIVA is depleted from these internalization events; thus, pSIVA should be added in excess to the culture medium during imaging experiments.

The use of pSIVA has been shown with various cell lines and primary neuronal cultures, including COS-7 cells⁶, HEK 293 cells (Y.E. Kim, J. Chen, J.R. Chan and R. Langen, unpublished data) and primary dorsal root ganglion (DRG) neurons⁶. In addition, pSIVA can be used with similar expectations in cells and conditions that have been investigated with other annexin-based methods^{1,4,9,11,12,22–25,27–30}. However, the technical limitations for continuous *in vivo* imaging at single-cell resolution remain challenging and untested. Currently, the application of pSIVA *in vivo* to examine neuronal degeneration has been shown only after transection or injury, in combination with static imaging on exposure of the nerve tract⁶. As technical advances in noninvasive fluorescence *in vivo* imaging continue to develop, we anticipate that the application of pSIVA will provide a means to monitor the apoptotic process in real time.

Experimental design

Selection of polarity-sensitive label. In this protocol, we describe the construction of pSIVA, an annexin B12 variant that is designed to fluoresce only when bound to PS and is compatible with the green fluorescence filters commonly equipped on fluorescence microscopes. To label the protein, we use IANBD

(*N,N'*-di-methyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethyleneamine), which has a green fluorescence profile similar to that of fluorescein isothiocyanate, with an excitation maximum at 478 nm and an emission maximum at 525 nm in the nonpolar lipid environment (540 nm in polar solution).

A blue fluorescent pSIVA variant can also be constructed by labeling annexin B12 with BADAN (6-bromoacetyl-2-dimethylaminonaphthalene), which has an excitation maximum at 380 nm and an emission maximum at 450 nm in the lipid environment (530 nm in solution). BADAN-labeled pSIVA variants can be used with typical blue fluorescent or DAPI (4',6-diamidino-2-phenylindole) filters, providing a tenfold increase in fluorescence intensity for the membrane-bound state compared with the solution state⁶. However, because there is a large blue shift in the fluorescence emission maximum for the fluorophore in solution (530 nm) as opposed to in a nonpolar environment (450 nm), BADAN-labeled pSIVA variants can be used with custom-designed filters that exclude most of the fluorescence from the solution state. For example, a custom-designed 420–470-nm bandpass filter will provide a ~50-fold increase in fluorescence intensity of the membrane-bound state in comparison with the solution state⁶.

Necessary precautions and controls. To ensure that cysteines at positions 101 and 260 do not oxidize before labeling, ensure that fresh dithiothreitol (DTT) is continuously present during purification (Steps 11–24) and during storage at –80 °C (Step 27). For gel filtration (Steps 22 and 23) during protein purification, fractions containing purified annexin B12 (L101C, L260C) protein can be identified by running an aliquot of the peak fractions on an SDS-PAGE gel, followed by Coomassie staining (see Step 23). Typically, the protein will be in the second peak, whereas the first peak (in the void volume) will contain residual lipids. DTT should be removed immediately before the labeling of annexin B12 (L101C, L260C) protein with the IANBD dye, using a PD-10 column (Step 25).

For live-cell imaging experiments using a six-well plate, at least a single well should be used as a negative control, in which DRG neurons are imaged with pSIVA and propidium iodide in normal growth medium. For experiments aimed at detecting rescue from neuronal degeneration, a second well should be used as a positive control and imaged with the presence of pSIVA and propidium iodide under continuous nerve growth factor (NGF) deprivation.

MATERIALS

REAGENTS

pSIVA purification

- Annexin B12 (L101C, L260C) plasmid containing cysteine mutations at residue positions 101 and 260 was constructed from the cysteine-less annexin B12 pSE420-mrp33H.09 plasmid³¹ (C113A and C302A) (plasmid available on request).
- Z-Competent DH5α *Escherichia coli* strain (Zymo Research, cat. no. T3007)
- Agar (Fisher, cat. no. BP1423-500)
- LB broth (BD Biosciences, cat. no. 240210)
- Bacto yeast (BD Biosciences, cat. no. 212720)
- Bacto tryptone (BD Biosciences, cat. no. 211699)
- Glycerol (JT Baker, cat. no. M778)
- Ampicillin (Sigma, cat. no. 9518)
- Isopropyl-β-D-thiogalactoside (IPTG; Promega, cat. no. V3953)
- Phenylmethylsulfonyl fluoride (PMSF; Sigma, cat. no. 7626)
- DTT (Roche, cat. no. 10709000001)
- Leupeptin (Sigma, cat. no. 62070)
- Aprotinin (Sigma, cat. no. 10820)
- CaCl₂ (Sigma, cat. no. C3306)

- EDTA (Sigma, cat. no. E5134)
- MgCl₂ (Sigma, cat. no. M0250)
- NaCl (MP Biomedicals, cat. no. 194848)
- KH₂PO₄ (Sigma, cat. no. P5655)
- K₂HPO₄ (Sigma, cat. no. S7907)
- Sucrose (Sigma, cat. no. S0389)
- Sodium azide (Sigma, cat. no. S8032) **! CAUTION** Toxic; wear gloves when handling.
- Phosphatidylserine, obtained from bovine brain (PS; Sigma, cat. no. P7769)
- Phosphatidylcholine, obtained from egg yolk (Sigma, cat. no. P3556)
- IANBD (Invitrogen, cat. no. D2004)
- DMSO (Sigma, cat. no. D8418)
- HEPES (free acid; MP Biomedicals, cat. no. 194848)
- NaOH pellets (EMD Chemicals, 1310-73-2)
- Dulbecco's PBS (D-PBS; Invitrogen, cat. no. 14190-144)
- Pierce BCA Protein Assay Kit (Thermo Scientific, cat. no. PI23227)
- Digitonin (Sigma, cat. no. D141)
- Precast 10% SDS-PAGE (Invitrogen, cat. no. WT0101A)

- 0.1% Coomassie blue solution (Invitrogen, cat. no. R-250)
- Propidium iodide (Invitrogen, cat. no. P3566)

Rat DRG purification

- Timed-pregnant rat (15 d gestation, Sprague-Dawley; Charles River Laboratories) **! CAUTION** Necessary approvals must be obtained; all animal procedures must be approved by an institutional animal care and use committee, and adhere to federal and state regulations.
- Minimal essential medium (MEM; GIBCO, cat. no. 11095-080)
- L15 medium (GIBCO, cat. no. 11415-064)
- Trypsin (0.25% (wt/vol); GIBCO, cat. no. 25200-056)
- Fetal bovine serum (FBS; GIBCO, cat. no. 11415-064, or Hyclone, cat. no. SH30070.03)
- FdU (fluorodeoxyuridine; 2–5 mM; Sigma, cat. no. F0503)
- N2 supplement (100×; GIBCO, cat. no. 17502-048)
- Nerve growth factor (NGF; Serotec, cat. no. PMP04Z)
- Collagen (purified from rat tails)
- TrkA-Fc (R&D Systems, cat. no. 1056-TK)
- Anti-NGF antibody clone 27/21 (Millipore)

EQUIPMENT

- Syringe (10 ml; BD Biosciences, cat. no. 301604)
- Syringe (3 ml; BD Biosciences, cat. no. 309586)
- Filter (0.45 µm; VWR, cat. no. 28143-312)
- Filter (0.2 µm; VWR, cat. no. 28143-310)
- Amicon ultra-15 centrifugal filter unit (10,000-Da molecular weight cutoff (MWCO); Millipore, cat. no. UFC901024)

Gel filtration setup

- HiLoad 26/60 Superdex (75 pg; GE Healthcare, cat. no. 17-1070-01)
- AKTApplc system (GE Healthcare)
- PD-10 column (GE Healthcare, cat. no. 17-0851-01)

Live-imaging setup

- Zeiss Axiovert 200 motorized inverted microscope
- Zeiss complete incubation system: XL-3 incubator Axiovert 200, temperature control unit 27-2, heating unit incubators, heating insert P scan stage, CO₂ cover HP heating insert P, heating insert M06 frame K, CO₂ cover HM heat insert M, CO₂ controller incubator XL-2, POC-R cell cultivation system
- Digital imaging system: Photometrics Cascade 1K camera, AV4 driver for Roper, AV4 driver for Uniblitz, external Uniblitz shutter, VCM-D1 controller, AxioCam MRM REV 3/Firewire, AV4 basic software, AV4 MOD MC/T/Z, AV4 MOD autofocus, AV4 MOD Mark and Find 2, Zeiss Imaging WS/20A high-end software

REAGENT SETUP

Ampicillin stock (1,000×) Dissolve 1 g of ampicillin powder in distilled H₂O to a final volume of 10 ml. Filter-sterilize and store 1-ml aliquots at –20 °C. These can be stored for several months at –20 °C.

LB medium Add 10 g of LB broth powder to 500 ml of distilled H₂O in a 2 liter flask. Autoclave at 121 °C for 15 min and let cool to room temperature (20–25 °C). Prepare fresh before use.

LB agar plates Add 15 g of agar and 10 g of LB broth powder and a stir bar to 500 ml of distilled H₂O in a 1-liter Pyrex bottle. Autoclave at 121 °C for 15 min and let cool to ~55 °C while stirring. Add 500 µl of the 100 µg ml⁻¹ ampicillin stock solution and allow to mix. Pour ~10 ml of the LB agar solution into 10-cm Petri dishes and allow to cool. Plates can be stored in a plastic sleeve at 4 °C for months.

TB medium Add 6 g of tryptone, 12 g of yeast extract and 2 ml of glycerol to 450 ml of distilled H₂O. Sterilize by autoclaving and cool to room temperature. Adjust the volume to 500 ml and add 1.16 g KH₂PO₄ and 6.27 g K₂HPO₄. Prepare fresh before use.

HEPES-NaCl buffer To prepare 10× HEPES-NaCl stock, dissolve 23.83 g of HEPES and 29.22 g of NaCl in 450 ml of water, and adjust the pH to 7.4 with NaOH pellets; adjust the volume to 500 ml with distilled H₂O and filter sterilize. To prepare 1× stock, dilute tenfold with distilled H₂O before use (final concentration is 20 mM HEPES containing 100 mM NaCl (pH 7.4)). Both 10× and 1× stocks can be stored for years at room temperature.

Sucrose solution (240 mM; + 0.1% (wt/vol) NaN₃ to prevent bacterial growth) To prepare 500 ml of stock, add 41.1 g of sucrose and 0.5 g of sodium azide to water and filter sterilize. This can be stored at 4 °C for several months to years.

EDTA solution (0.5 M; pH 8.0) Dissolve 93.05 g of EDTA powder in 400 ml of distilled H₂O. Stir vigorously using a magnetic stirrer, while adjusting pH to 8.0 with ~10 g of NaOH pellets. It will not dissolve until a pH of 8.0 is reached. Adjust the final volume to 500 ml with distilled H₂O and filter sterilize. This can be stored for years at room temperature.

CaCl₂ solution (1 M) Dissolve 14.7 g of CaCl₂ in 80 ml of distilled H₂O, adjust the final volume to 100 ml with distilled H₂O and filter sterilize. This can be stored for years at room temperature.

MgCl₂ solution (1 M) Dissolve 20.33 g of MgCl₂ in 80 ml of distilled H₂O, adjust the final volume to 100 ml with distilled H₂O and filter sterilize. This can be stored for years at room temperature.

Lipid vesicles To prepare a 50 mg ml⁻¹ solution of PS-containing membranes, mix equal amounts of PS with phosphatidylcholine lipid powder and vortex in 4 ml of 240 mM sucrose (use ~200 mg of total lipid (PS and phosphatidylcholine) per cell pellet from 1 liter of bacterial culture). Vortexing the lipid powder mixture of phosphatidylcholine and PS in sucrose will result in a crude preparation of sucrose-filled unilamellar and multilamellar vesicles that is appropriate for protein purification. These vesicles should be prepared fresh before use.

Annexin purification buffer Prepare a buffer solution containing 4 mM MgCl₂, 2 mM DTT and 1 mM EDTA in HEPES-NaCl buffer (see above). To prepare 200 ml, mix 20 ml of 10× HEPES-NaCl stock solution with 800 µl of 1 M MgCl₂, 400 µl of 1 M DTT and 400 µl of 0.5 M EDTA. Adjust the volume to 200 ml with distilled H₂O. The buffer should be prepared fresh before use.

M1 DRG medium M1 medium consists of MEM containing high glucose, 100 ng ml⁻¹ of NGF and 10% FBS (Hyclone). Medium is prepared by combining 100 ml of MEM with 10 µl of NGF and 10 ml of FBS. Maximum recommended shelf life is 1 month at 4 °C.

E2F antimetabolic medium E2F medium consists of MEM containing high glucose, antimetabolic FdU (2–5 mM), N2 supplement (1:100 dilution of 100× solution) and 100 ng ml⁻¹ of NGF. Medium is prepared by combining 100 ml of MEM with 500 µl of FdU, 1 ml of N2 supplement and 10 µl of NGF. Maximum recommended shelf life is 1 month at 4 °C.

Rat-tail collagen type I Rat-tail collagen was purified as previously described³⁰.

PROCEDURE

Transformation of DH5α *E. coli* with the annexin B12 (L101C, L260C) plasmid ● **TIMING** 1 d (15 min plus overnight incubation at 37 °C)

- 1| Thaw Z-Competent DH5α cells on ice.
- 2| Pipette 50 µl of thawed cells into a prechilled 1.5-ml microcentrifuge tube.
- 3| Add 1 µl of the annexin B12 (L101C, L260C) plasmid (0.1–0.2 mg ml⁻¹ of plasmid concentration).
- 4| Incubate the mixture on ice for 5 min (maximum 60 min).
- 5| Spread the transformation mixture on a prewarmed LB agar plate containing 100 µg ml⁻¹ of ampicillin and incubate the plates at 37 °C overnight.

Protein expression ● TIMING 2 d

- 6| Inoculate 5 ml of LB medium containing 100 $\mu\text{g ml}^{-1}$ (5 μl of 1,000 $\times$ stock) of ampicillin (LB-amp) with a bacterial colony from the overnight transformation, and incubate in a 37 °C shaker (225 r.p.m.) for ~8 h.
- 7| Transfer 100 μl of the culture into a 250-ml flask containing 50 ml of LB-amp and grow overnight in a 37 °C shaker (225 r.p.m.).
- 8| In the morning, dilute 25 ml of the culture into two 2-liter flasks, each containing 500 ml of TB medium supplemented with 100 $\mu\text{g ml}^{-1}$ of ampicillin. Incubate in a 37 °C shaker for ~5–6 h or until an optical density (OD) of ~1.3–1.5, as determined by spectrophotometry, is reached (colonies cultured on LB can only grow up to an OD₆₀₀ of 0.6–0.8).
- 9| To induce protein expression, add IPTG to a 0.5 mM final concentration (500 μl of a 0.5 M stock) and incubate in the shaker (150 r.p.m.) at 22 °C overnight.

Protein purification ● TIMING 6–8 h

- 10| Pellet the cultures for 20 min at 4 °C and 6,000*g*.
- 11| Remove the supernatant and resuspend each cell pellet in annexin purification buffer (up to 50 ml).
- 12| Add 500 μl of 1 M PMSF, 500 μg of leupeptin and 500 μg of aprotinin.
- 13| Lyse cells by sonication at 8–10 W, on ice, for ~3 $\times$ 10 min. Centrifuge at 4 °C and 9,000*g* for 20 min.
- 14| Transfer the supernatant to clean tubes (discarding the pellet).
- 15| Add ~135 μl of 1 M CaCl_2 to ~45 ml volume of the supernatant in each tube, to prepare a 3 mM final CaCl_2 concentration.
- 16| Add 200 mg of lipid vesicles to the supernatant in each tube.
- 17| Mix by inverting the tubes four or five times, incubate for 5 min and centrifuge for 15 min at 4 °C and 20,000*g*.
- 18| Decant the supernatant (collect it and keep aside just in case; see Step 23 of Troubleshooting table), and wash the lipid pellets by resuspending in 25 ml of annexin purification buffer supplemented with 3 mM CaCl_2 and pelleting by centrifuging at 20,000*g* for 15 min at 4 °C. Repeat this step.
- 19| Resuspend the lipid pellet with bound protein in annexin purification buffer supplemented with 5 mM EDTA (75 μl of a 1 M stock) to a final total volume of 10 ml.
- 20| Centrifuge at 20,000*g* for 1 h at 4 °C.
- 21| Annexin B12 (L101C, L260C) protein is in the supernatant. Decant the supernatant and filter through a 0.45- μm syringe filter into a clean 15- or 50-ml conical tube.
- **PAUSE POINT** After this step, the protein can be stored, with fresh DTT (1–2 mM; 0.15–0.30 mg l^{-1}) added to it, at 4 °C for further purification by gel filtration the next day.
- 22| To further purify the protein by size exclusion chromatography (SEC), load 10 ml of annexin B12 protein supernatant onto a Superdex 75 column (prep grade, 26/60), pre-equilibrated with two column volumes (640 ml) of HEPES-NaCl buffer (pH 7.4), containing 1 mM EDTA, and 1 mM DTT. Solution is made fresh by diluting 100 ml of 10 $\times$ stock of HEPES-NaCl, 2 ml of 0.5 M EDTA stock and 0.154 g DTT. Bring final volume to 1 liter. For detailed instructions on SEC, refer to the manufacturer's instruction handbook (GE Healthcare Life Sciences, cat. no. 56-1190-99 AE).
- 23| Collect the eluate from the Superdex 75 column and check for peak fractions containing annexin B12 (L101C, L260C) protein. Determine the purity by SDS-PAGE and Coomassie blue staining (annexin B12 is a 36-kDa protein). For detailed instructions on the principles of protein loading and elution from SEC, refer to the manufacturer's instruction handbook (GE Healthcare Life Sciences, cat. no. 56-1190-99 AE).

? TROUBLESHOOTING

- 24| Concentrate the fractions containing annexin B12 with a 15-ml centrifugal filter (MWCO 10,000 Da) according to the manufacturer's instructions (Amicon Ultra-15; Millipore).

25| Remove DTT and exchange the buffer using a PD-10 column and elute the protein in HEPES-NaCl buffer without DTT, or alternatively in D-PBS according to the column manufacturer's instructions (GE Healthcare).

26| Calculate protein concentration (c) by measuring UV absorbance at 280 nm (A_{280}) with a spectrophotometer and using the following equation:

$$(c = A_{280} / \epsilon \cdot l) \cdot MW$$

using the extinction coefficient $\epsilon = 12,287.8 \text{ l mol}^{-1} \text{ cm}^{-1}$, where l is the path length (cm) of the cuvette, and the molecular weight (MW) of annexin B12 is 35,108.

27| Aliquot 500 μg of protein for labeling and add 1 mM (0.154 mg l^{-1}) DTT to the remainder of the protein. Place 500- μg aliquots of the protein into 1.5-ml microcentrifuge tubes for later use and store at -80°C .

■ **PAUSE POINT** The unlabeled protein can be stored indefinitely with DTT at -80°C .

Labeling annexin B12 (L101C, L260C) protein with IANBD ● **TIMING 2.5 h–1 d**

28| Prepare a 10 mM stock of IANBD by dissolving 0.4 mg in 100 μl of DMSO.

▲ **CRITICAL STEP** Keep the IANBD stock protected from light by covering in foil.

29| Label the 500- μg aliquot of the protein ($\sim 28 \text{ nmol}$) in D-PBS (with DTT removed) by adding a tenfold molar excess of IANBD ($\sim 280 \text{ nmol}$; 28 μl of the 10mM stock). Protect from light by covering the tube with foil, and incubate for $\sim 2 \text{ h}$ at room temperature ($20\text{--}25^\circ\text{C}$) or overnight at 4°C .

30| Quench the labeling reaction with a twofold molar excess of β -mercaptoethanol ($\sim 560 \text{ nmol}$; 40 μl of a 1,000 $\times$ dilution of 14M β -mercaptoethanol), and elute labeled proteins with a PD-10 column containing HEPES-NaCl buffer (or, alternatively, D-PBS). PD-10 columns are packed with Sephadex G-25 medium.

! **CAUTION** High concentrations of HEPES can be toxic to some cell types.

31| Determine the degree of labeling by measuring absorption (A_x) at 478 nm and using an extinction coefficient (ϵ) of $25,000 \text{ M}^{-1} \text{ cm}^{-1}$ for IANBD and a MW of 35,108 Da for the annexin B12 (L101C, L260C) protein using the following formula:

$$(A_x / \epsilon) \times (\text{MW of protein} / \text{mg protein ml}^{-1}) = (\text{moles of dye} / \text{moles of protein})$$

The concentration of labeled protein can be determined using a BCA assay kit (Pierce), following the manufacturer's instructions. The protein should be $\sim 99.9\%$ labeled, with a stoichiometry of 2 mol of IANBD to 1 mol of annexin B12 (L101C, L260C).

32| Filter the labeled protein (pSIVA) through a 0.2- μm syringe filter into an autoclaved 1.5-ml microcentrifuge tube, and store at room temperature ($20\text{--}25^\circ\text{C}$) or at 4°C . Keep protected from light by wrapping the tube with foil.

Purification, growth and maintenance of DRG neurons³⁰ ● **TIMING 10 d**

33| Coat tissue culture-treated plastic or glass coverslips with rat-tail collagen 2–3 d before dissection. Dilute the collagen in sterile water to a concentration of 0.1 mg ml^{-1} . The standard protocol in coating wells or coverslips includes preparing a gel layer of collagen; however, an alternative to this approach is to just add a thin monolayer of collagen onto the coverslips and let them dry (between 48 and 72 h in a hood).

34| Obtain timed-pregnant (E15) rat or mouse and dissect out the spinal cords and individually remove DRGs under sterile conditions. Place DRGs in a 15-ml conical centrifuge tube with 5 ml of L15 medium + 10% heat-inactivated FBS and allow them to settle.

35| Remove the L15 medium + FBS and wash DRGs with 10 ml of L15 medium without serum. Allow DRGs to settle.

36| Replace L15 with 5 ml of 0.25% trypsin and incubate DRGs in a 37°C water bath for 45 min.

37| Remove as much trypsin as possible and wash DRGs with 10 ml of L15 + 10% FBS.

38| Allow DRGs to settle or centrifuge gently at 250g for 10 min.

39| Remove the supernatant and replace with 4–5 ml of M1 DRG medium.

40| Triturate the ganglia with a fire-polished Pasteur pipette until the suspension is homogeneous.

41| Plate dissociated DRGs onto collagen-coated tissue culture-treated plastic or collagen-coated glass coverslips (from Step 33). Plate at a density of one spinal cord per six-well culture dish (25-mm coverslips) or ~150,000 total cells per well at a volume of ~200 μ l. Do not flood the wells with medium. Incubate at 37 °C, 5% CO₂.

▲ CRITICAL STEP Experiments should be performed on tissue culture-treated plastic. However, if using glass coverslips, they must be attached to culture wells before plating cells to prevent coverslips from shifting during imaging. This can be accomplished using various nontoxic adhesives (silicon caulk, cyanoacrylate) or even by using a drying substrate (collagen) with the coverslips in wells to allow attachment.

42| The next day, flood the wells with 1 ml of E2F medium.

43| After 2 d, remove 1 ml of E2F medium and add 1 ml of M1 DRG medium. Continue the feeding cycle by feeding cells with M1 DRG medium or E2F medium alternately every 2 d. After three full cycles, maintain cells in 1 ml of M1 DRG medium and feed with 1 ml M1 DRG medium every 3 d.

44| Elaborate neurites will extend throughout the culture dish and can be used for experiments after ~8–10 d.

▲ CRITICAL STEP For NGF-deprivation experiments, neurons ideally should be used after 1–2 weeks. After 3 weeks in culture, the neurons will be independent of NGF for survival.

Time-lapse imaging protocol ● TIMING 2 d

45| Turn on the fluorescence lamp, incubation system (37 °C, 5% CO₂ in a humidified atmosphere), motorized stage, microscope and camera, and let the system equilibrate at 37 °C for ~2–3 h.

▲ CRITICAL STEP This is an essential step for time-lapse imaging experiments using an automated system with programmed positions and focus points, as slight changes in temperature in the system will disrupt the focus.

46| Take a six-well plate of purified DRG neuronal cultures that are still dependent on NGF for survival (cultured ~1–2 weeks; from Step 44).

47| Initiate degeneration in specific wells by removing NGF from the culture medium. Wash cells three times with M1 DRG medium without NGF, or alternatively wash once and add 1 μ g ml⁻¹ of anti-NGF antibody clone 27/21 or 1 μ g ml⁻¹ of the fusion protein TrkA-Fc (1 μ l of a 1 μ g μ l⁻¹ solution to 1 ml of medium) in the absence of NGF.

48| Always use one well for a negative control and replace the medium with M1 DRG medium (containing NGF).

49| Add 5–10 μ g ml⁻¹ pSIVA (from Step 32) and 0.3 μ g ml⁻¹ propidium iodide to each well.

50| Secure the six-well plate on the microscope stage.

▲ CRITICAL STEP The plate temperature should also be equilibrated in the microscope incubation chamber ~1 h before imaging.

51| Using the microscope software, set up the experimental program as follows: time course of imaging and time interval for capturing images; for 1-week-old DRG neurons deprived of NGF, the time course of degeneration is 24–40 h, and an interval of ~30 min is appropriate.

52| Set fluorescence channels and exposure times: for pSIVA, use the green fluorescence filter set (excitation maximum 487 nm and emission maximum 525 nm).

▲ CRITICAL STEP For extended time-lapse imaging experiments, bleaching should be minimized 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). Particular attention should be paid for experiments aimed at imaging the reversal of pSIVA binding, which is indicative of rescue from degeneration and apoptosis.

53| Pick the positions and focal depth for the different fields of view to be imaged.

54| Before starting the time-lapse imaging experiment, recheck the positions and focus for each field of view.

55| Start the time-lapse experiment.

? TROUBLESHOOTING

56| Analyze and confirm results at the end of the experiment as described previously⁶.

● TIMING

Steps 1–5, Transformation of DH5α *E. coli* with the annexin B12 (L101C, L260C) plasmid: 1 d (15 min plus overnight incubation at 37 °C)

Steps 6–9, Protein expression: 2 d

Steps 10–27, Protein purification: 6–8 h

Steps 28–32, Protein labeling: 2.5 h–1 d

Steps 33–44, Purification, growth and maintenance of DRG neurons: 10 d

Steps 45–56, Time-lapse imaging: 2 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Step 23	No 36-kDa annexin B12 band	Annexin was not bound to membranes in Step 17	Check the supernatants from Step 18 and repeat binding and washing Steps 14–23, making sure the Ca ²⁺ concentration is 3-fold higher than the EDTA concentration
		Annexin is still bound to the lipid Step 21	Take the lipid pellet from Step 21 and repeat Steps 19–23, making sure the EDTA concentration is ≥5 mM
Step 55	No pSIVA fluorescence	Annexin B12 (L101C, L260C) was not properly labeled with IANBD due to cysteine oxidation, incomplete removal of DTT in Step 25, or bleached IANBD stock	Make sure the pSIVA stock is properly labeled by testing pSIVA fluorescence with cells that have been permeabilized with 0.02% digitonin. Alternatively, perform an <i>in vitro</i> membrane binding assay ⁶ , comparing the fluorescence intensities of the bound and unbound states. If the protein is not labeled, make sure fresh DTT was used in purification Steps 10–24 and repeat Steps 25–31
		Ca ²⁺ is not present in the culture medium	Check medium formulations and ensure that the Ca ²⁺ (CaCl ₂) concentration is ~1–2 mM
		Suboptimal microscope settings	Ensure 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

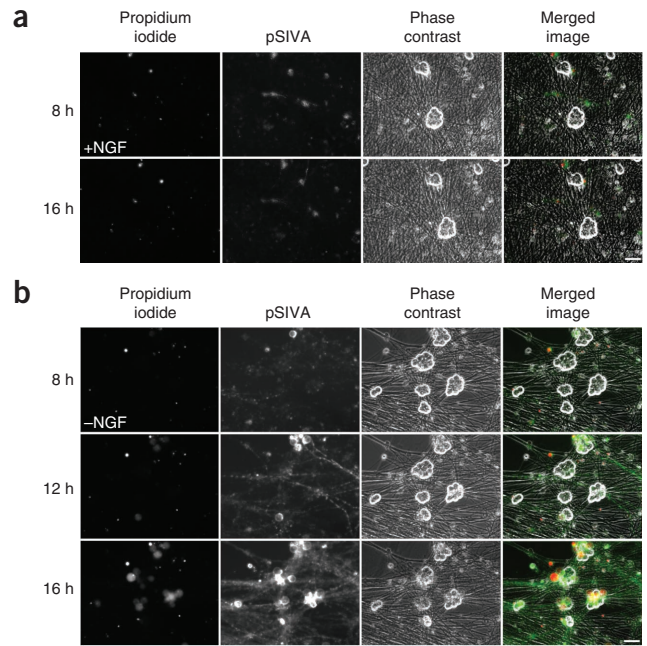
ANTICIPATED RESULTS

Using the protocol described above for bacterial expression of annexin B12 (L101C, L260C), a 1-liter culture produces ~15 mg of total protein, which can be stored in 500-μg aliquots at –80 °C for later use. Protein purity can be checked by SDS-PAGE and Coomassie blue staining, and should be ~99.9% pure (see Step 23). The IANBD-labeled annexin B12 (L101C, L260C), known as pSIVA, is stable for several months and can be stored protected from light at room temperature (20–25 °C) or at 4 °C. The labeling reaction is performed with a tenfold molar excess of the IANBD dye to the annexin B12 protein, and the expected stoichiometry for labeled pSIVA is 2 mol of IANBD dye to 1 mol of annexin B12 (L101C, L260C) protein (see Step 31 and **Fig. 1**).

During the initial stages of apoptotic processes, PS is translocated from the inner leaflet to the outer leaflet of the plasma membrane. The progression of degeneration in DRG neurons is imaged using pSIVA to track PS exposure on the external leaflet of the plasma membrane over 1–2 d (**Fig. 2**). In 1-week-old DRG neurons that are induced to undergo

Figure 2 | Application of pSIVA for monitoring the degeneration of DRG neurons. **(a,b)** Time-lapse microscopy was used to image DRG neurons in normal survival conditions **(a)** and after NGF deprivation **(b)**. The left panels show propidium iodide fluorescence, pSIVA fluorescence and phase contrast images, and the right panels display the merged images. In the merged images, propidium iodide is represented by red fluorescence and pSIVA by green fluorescence, both of which are overlaid onto the phase contrast images. The hours shown indicate the time after NGF was removed from the culture medium **(b)** or replaced with fresh medium **(a)**. Scale bars, 100 μ m.

degeneration, pSIVA fluorescence and binding to localized areas of PS exposure on the axon can be detected as early as ~10 h, and progressively increases over time along the axon in either direction (toward the cell body or axon terminals). pSIVA staining of the cell body occurs later, and is followed by loss of membrane integrity and propidium iodide staining of the cell body. Previously, we also observed cell-cell variability and heterogeneity in the initiation of pSIVA fluorescence in degenerating sensory neurons under these conditions⁶.



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AUTHOR CONTRIBUTIONS R.L. conceived the study and, together with J.C. and J.R.C., supervised the project. Y.E.K. designed and performed the experiments and, together with J.R.C., designed and performed the experiments involving neurons. R.L., J.C., J.R.C. and Y.E.K. analyzed the data. Y.E.K. and J.R.C. wrote the paper.

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