

Engineering a polarity-sensitive biosensor for time-lapse imaging of apoptotic processes and degeneration

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Apoptosis is of central importance to many areas of biological research, but there is a lack of methods that permit continuous monitoring of apoptosis or cell viability in a nontoxic and noninvasive manner. Here we report the development of a tool applicable to live-cell imaging that facilitates the visualization of real-time apoptotic changes without perturbing the cellular environment. We designed a polarity-sensitive annexin-based biosensor (pSIVA) with switchable fluorescence states, which allows detection only when bound to apoptotic cells. Using pSIVA with live-cell imaging, we observed dynamic local changes in individual rat neurons during degeneration *in vitro* and *in vivo*. Furthermore, we observed that pSIVA binding was reversible and clearly defined the critical period for neurons to be rescued. We anticipate pSIVA can be widely applied to address questions concerning spatiotemporal events in apoptotic processes, its reversibility and the general viability of cells in culture.

Live-cell imaging has become a valuable technique for studying dynamic biological processes in real time. The ability to visualize and track active processes in a single living cell has provided insights into cellular architecture, membrane organization, dynamic protein assemblies, molecular organization and cellular responses to external stimuli.

Central to our understanding of cellular and pathological processes is the knowledge of the apoptotic state of the cells of interest. Whereas the morphological¹ and biochemical changes that occur at different stages of apoptosis are well understood, imaging these changes in living cells has been difficult. Several assays are available, which are aimed at detecting the specific biochemical changes that occur at different stages of apoptosis, such as, phosphatidylserine (PS) exposure to the outer leaflet of the plasma membrane^{2,3}, mitochondrial dysfunction⁴, activation of caspases⁵, DNA fragmentation⁶ and loss of membrane integrity^{3,7}. Current methods for these assays are limiting and generally disruptive to the cellular environment. In most cases, these assays are toxic to the cells or require fixation.

Detection of PS on the extracellular face of the plasma membrane is an attractive target for live-cell imaging for several reasons. In healthy cells, plasma membrane asymmetry is closely regulated, and PS is restricted to the inner leaflet⁸. Exposure of PS has been well established as a near-universal indicator of early apoptotic processes^{2,7}. Also, PS provides extracellular binding targets that can be detected without the need to penetrate the cell. Moreover, it is an early event in apoptosis^{7,9,10}; thus, monitoring PS exposure provides a way to observe the initiation of the apoptotic pathway before other changes are present. This is particularly useful for the detection of early degenerative processes for the cases in which there is no progression to cell death (that is, neuronal pruning or Wallerian degeneration).

The current method for PS detection involves using a fluorescently tagged annexin protein to detect PS exposed on the plasma membrane of cells that have initiated apoptosis³. The annexins are widely used in apoptosis assays because of their ability to bind specifically to negatively charged lipids such as PS¹¹. The different annexin types have a variable N-terminal tail and a structurally conserved core domain. The core domain comprises the annexin repeat, which contains five α -helices, designated A–E. The calcium-binding loops are located between the A–B and D–E helices on the convex side of the protein and mediate membrane binding^{11–13} (**Fig. 1a**).

The annexin-based probes described to date are impractical for live-cell imaging experiments because separate steps are required for binding of the fluorescent annexin probe to the apoptotic cells and subsequent removal of the unbound protein to reduce the background fluorescence before analysis by microscopy. To circumvent these problems, we engineered an annexin-based fluorescent biosensor with built-in ‘on’ (membrane-bound) and ‘off’ (in solution) fluorescent states. Based on our previous studies on the solution and Ca^{2+} -dependent membrane-bound structures of annexin B12 (anxB12; also known as annexin XII)^{12–14}, we conjugated polarity-sensitive thiol-reactive fluorophores to cysteines introduced at specific sites in annexin. This coupled membrane binding to a measurable increase in fluorescence emission intensity.

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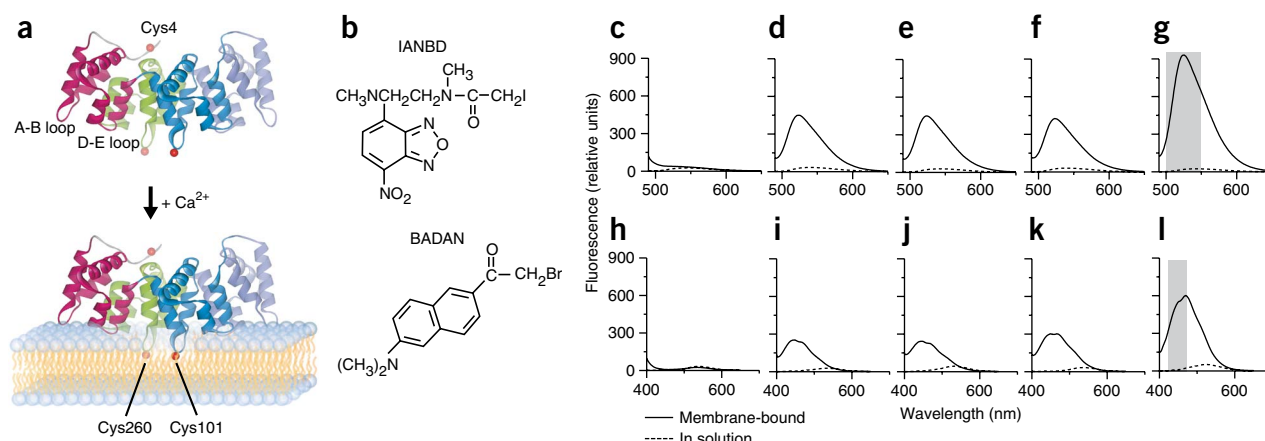


Figure 1 | Structure-based design and fluorescence properties of annexin-based pSIVA biosensors. **(a)** Crystal structure of anxB12 (Protein Data Bank entry 1AEI)²⁵ in solution with the four annexin repeats, represented by different colors. Each repeat contains two calcium- and membrane-binding loops: A-B and D-E loops (labeled for repeat I). A representation of anxB12 interaction with negatively charged membranes in the presence of Ca^{2+} is shown with the location of tested sites (red spheres on C_α) in the membrane binding loops (Cys101, Cys260) used to create pSIVA. **(b)** Structures of polarity-sensitive labels *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) and 6-bromoacetyl-2-dimethylaminonaphthalene (BADAN). **(c–l)** Fluorescence emission intensities (excitation wavelength was 478 nm for IANBD-labeled annexins and 380 nm for BADAN-labeled annexins) of in-solution and membrane-bound anxB12 Cys4-IANBD (**c**), anxB12 Cys101-IANBD (**d**), anxB12 Cys260-IANBD (**e**), anxA5 Cys262-IANBD (**f**), anxB12 Cys101,Cys260-IANBD (**g**; shaded area denotes the emission profile of a typical filter set for green fluorescence), anxB12 Cys4-BADAN (**h**), anxB12 Cys101-BADAN (**i**), anxB12 Cys260-BADAN (**j**), anxA5 Cys262-BADAN (**k**) and anxB12 Cys101,Cys260-BADAN (**l**; shaded area denotes the ideal emission profile, 420–470 nm, which may be used for the design of custom filters).

We hypothesized that this polarity-sensitive indicator of viability and apoptosis (pSIVA), could be applicable to the investigation of real-time, chronological and dynamic events occurring in apoptosis by live-cell imaging. One of the advantages of live-cell imaging methods is the ability to observe the distinct cell-to-cell variations in the responses to the apoptotic stimulus, directly revealing the different vulnerabilities of individual cells. Along these lines, a better understanding of where the first cellular responses occur, the timing and the severity of the response is fundamental to the understanding of the pathophysiology of apoptotic processes. Moreover, pSIVA can be used as a tool to measure the viability of cells without perturbing experimental conditions.

We tested pSIVA in combination with time-lapse microscopy, to visualize and monitor the progression of the apoptotic pathway from early stages to complete cell death at the single-cell level. We first tested the efficacy of pSIVA in COS-7 cells with the addition of the apoptotic factor etoposide. To better approximate the physiological condition, we used pSIVA to image degeneration in primary dorsal root ganglion (DRG) sensory neurons, in which we induced cell death by nerve growth factor (NGF) deprivation, and observed a dynamic progression of degeneration, first in the axons and then in the cell bodies. In addition, we used pSIVA to visualize degenerating sciatic nerves *in vivo*. Also, we determined the critical period necessary for pSIVA-stained neurons to be rescued by replenishing NGF in the culture medium at various time points after deprivation and observed that pSIVA binding was reversible as neurons recovered and regained health.

RESULTS

Structure-based design of a polarity-sensitive biosensor

To design a probe more suited for live-cell imaging applications, we engineered annexin-based biosensors based on the structure of the Ca^{2+} -dependent membrane-bound state of anxB12 (refs. 12,13). We placed polarity-sensitive labels in the loop

regions that mediate Ca^{2+} -dependent membrane interactions, transitioning from a polar (aqueous solution) to a nonpolar (lipid membrane) environment upon membrane binding^{12,14} (Fig. 1a). We labeled residues at positions 101 and 260 owing to their ideal location in the membrane-binding loops (Fig. 1a). In addition to anxB12 labeled with a single polarity-sensitive fluorophore, we generated a double-labeled anxB12 molecule with thiol-reactive fluorophores attached via cysteines introduced at positions 101 and 260 in cysteine-less annexin variants, to increase the intensity of the probe. Because annexin A5 (anxA5; also known as annexin V) has already been widely used and characterized for apoptosis assays^{3,15}, we also tested position 262 in anxA5, a site homologous to residue 260 in anxB12 (ref. 11). As a negative control, we labeled residue 4 in the N-terminal tail on the concave side of anxB12, which we expected to stay fully exposed to the aqueous environment in both the solution and membrane-bound states, to confirm that detected changes in fluorescence were directly a result of membrane interaction (Fig. 1a).

We screened for polarity-sensitive molecules that emit increased fluorescence intensity in nonpolar environments and chose two thiol-reactive labels: *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) and 6-bromoacetyl-2-dimethylaminonaphthalene (BADAN) (Fig. 1b).

Validation by *in vitro* fluorescence assays

To determine the differences in fluorescence emission between the solution and membrane-bound states, we measured the fluorescence intensities of these annexin-based polarity-sensitive biosensors in an *in vitro* binding assay. As predicted, the fluorescence intensity was negligible for all the labeled annexins free in solution (Fig. 1c–l), and for anxB12 Cys4-IANBD (Fig. 1c) and anxB12 Cys4-BADAN (Fig. 1h) in both in-solution and membrane-bound states. For anxB12 Cys101-IANBD, anxB12 Cys260-IANBD, anxA5 Cys262-IANBD we measured considerable

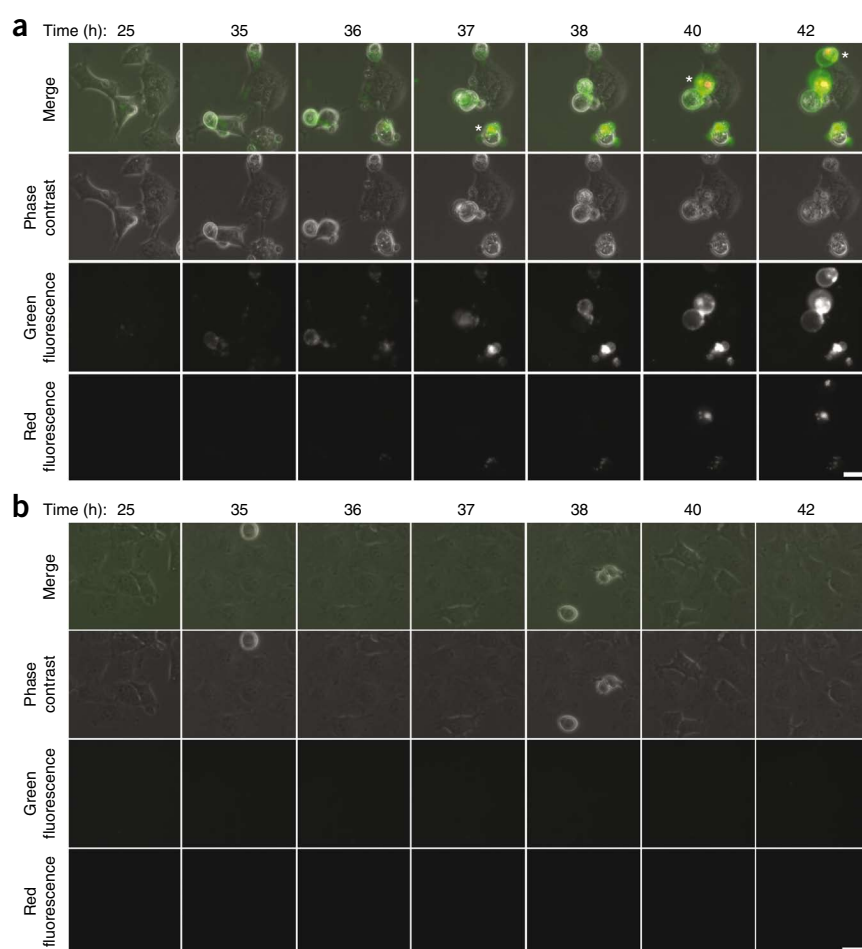


Figure 2 | Application of pSIVA_m to real-time live-cell imaging of apoptosis in COS-7 cells. **(a,b)** Time-lapse microscopy analysis of the same fields of view of COS-7 cells over 2 d in the presence of 100 μ M etoposide **(a)** and under physiological conditions without an apoptosis-inducing factor (DMSO, negative control; **b**). Cells were imaged with pSIVA_m and PI present in the culture medium for the duration of the experiment. Phase contrast, green fluorescence (pSIVA_m) and red fluorescence (PI) channels as well as merged images are shown. Green fluorescence indicates pSIVA_m binding to the PS exposed on the outer leaflet of the plasma membrane, and red fluorescence indicates PI staining of nuclei in cells in late apoptosis, with loss of plasma membrane integrity. Asterisks indicate when PI staining was first seen in the cell. Scale bars, 40 μ m.

Cys101,Cys260-BADAN compared to the in-solution state (**Fig. 1i**). For the various annexin-based probes tested, there was no detectable decrease in membrane binding or loss of specificity to PS (**Supplementary Fig. 1a–j**). We named our polarity-sensitive annexin-based biosensors pSIVAs to distinguish them from conventional fluorescence annexin-based sensors. We demonstrated the advantage of pSIVA over the conventional FITC-labeled anxA5 by measuring the respective fluorescence intensities in the presence of PS-containing vesicles (**Supplementary Fig. 1k**). As expected, the fluorescence intensities

increases in fluorescence intensities along with a slight blue shift from an emission maximum at 540 nm in the solution state to 525 nm in the membrane-bound state (**Fig. 1d–f**). Fluorescence emission measured for anxB12 labeled at both residue positions 101 and 260 with IANBD (anxB12 Cys101,Cys260-IANBD; referred to as pSIVA_m) in the membrane-bound state was substantially brighter, with only a negligible increase in the background fluorescence of the solution state (**Fig. 1g**). Based on the typical emission profiles of conventional filter sets for fluorescein isothiocyanate (FITC) or green fluorescence, we quantified the fluorescence emissions between 500 and 550 nm, revealing a ~45-fold increase in fluorescence of membrane-bound pSIVA_m when compared to the in-solution state (**Fig. 1g**).

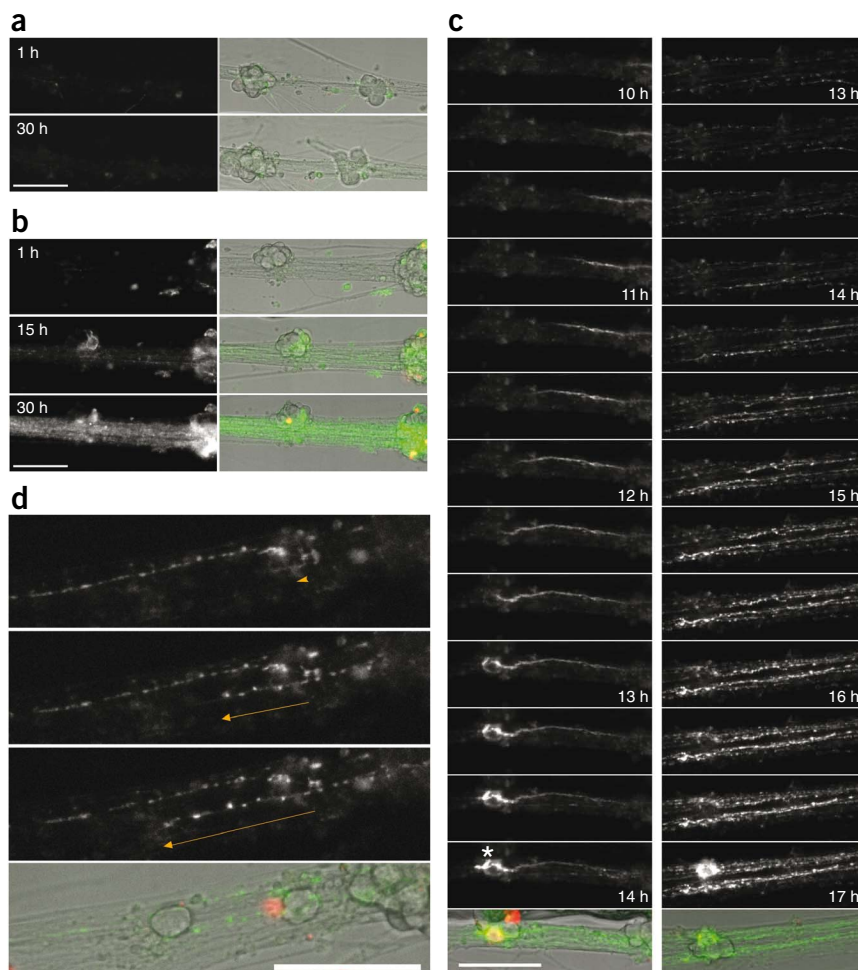
We measured slightly lower fluorescence intensities for the BADAN-labeled annexins at corresponding sites (**Fig. 1i–l**). Using the typical emission profile for blue fluorescence filter sets, we quantified fluorescence emissions between 400 and 650 nm, resulting in a ~10-fold increase in fluorescence intensity for the membrane-bound anxB12 Cys101,Cys260-BADAN compared to the solution state. We observed a large blue shift from an emission maximum of 530 nm for the in-solution state to 450 nm for the membrane-bound state, which may be used to design custom filters, to cut off most of the background fluorescence of the in-solution state while maximizing the emitted fluorescence of the membrane-bound state. For example, an analysis of the fluorescence emission intensities between 420 and 470 nm resulted in a ~50-fold increase in fluorescence of the membrane-bound anxB12

measured for the conventional FITC-labeled anxA5 remained the same regardless of whether the protein was in the in-solution or membrane-bound state, whereas pSIVA fluorescence strongly increased with increasing amounts of PS-containing membranes. Thus, attachment of polarity-sensitive labels IANBD and BADAN to residues in the membrane-binding loops provided an effective way to generate annexin derivatives with built-in 'on' and 'off' fluorescence states. Additionally, both IANBD- and BADAN-labeled annexins (green and blue fluorescence, respectively) may be used with conventional filter sets equipped on most fluorescence microscopes.

Application to live-cell imaging

To test the application of pSIVA to live-cell imaging and the capacity to specifically highlight cells undergoing the apoptotic pathway, we added pSIVA directly to the culture medium of COS-7 cells that we had induced to undergo apoptosis by etoposide¹⁶. We monitored the cells under physiological conditions (37 °C, 5% CO₂) by time-lapse microscopy. We tested both IANBD- and BADAN-labeled variants of pSIVA and observed that all the variants highlighted apoptotic cells (data not shown). Therefore, we used pSIVA_m for all subsequent cell culture experiments because of its enhanced fluorescence intensity compared to that of other pSIVA variants (**Fig. 1g**). IANBD also has the advantage of being excitable in the visible light spectrum, thereby avoiding the potentially harmful UV-light spectrum. As expected, we observed bright pSIVA_m staining of

Figure 3 | Application of pSIVA_m to monitoring the degeneration of DRG neurons. (a,b) Time-lapse microscopy analysis of DRG neurons under normal physiological conditions (a) and under NGF deprivation (b). Cells were imaged with pSIVA_m and PI present in the culture medium for the duration of the experiment. pSIVA_m fluorescence (left) and merged images of phase contrast, green (pSIVA_m) and red (PI) fluorescence (right) channels are shown. (c) Time-lapse images (3 frames per hour) showing the progressive movement of apoptotic PS exposure along single axons to cell bodies from 10 to 14 h after NGF removal in one area (left) and from 13 to 17 h in another area (right). Asterisk marks when PI staining was first seen in the cell body. (d) Localized progression of PS exposure along a single axon shown at 20-min intervals revealing a punctate staining pattern of pSIVA_m. Arrowhead indicates the starting point and arrows indicate the progression of PS exposure. Images at the bottom of c and d are merge of phase contrast, green (pSIVA_m) and red (PI) fluorescence of the last time point shown. The times shown indicate the time after NGF was removed (b–d) or replaced (a) in fresh medium. Scale bars, 100 μ m.



COS-7 cells in the early stages of apoptosis (Fig. 2a and Supplementary Fig. 2b) and a gradual increase in staining concurrent with progression into late-stage cell death, detected by propidium iodide (PI) staining. In comparison, we observed no annexin or PI staining in COS-7 cells grown under normal conditions (Fig. 2b and Supplementary Fig. 2a), confirming that pSIVA_m binding and fluorescence emission was specific to apoptotic cells. Furthermore the background fluorescence from the in-solution state was undetectable. To confirm that the presence of pSIVA_m in the culture medium did not perturb the cellular environment, we

grew COS-7 cells in the presence and absence of pSIVA_m and did not observe any differences in the cell growth rate (data not shown). Thus, the use of pSIVA_m in combination with live-cell imaging provides a means to continuously monitor the progression of apoptosis in living cells without perturbing the cellular environment.

Application to neuronal degeneration

Having established the utility of pSIVA_m in live-cell imaging of a simple model system, we next determined whether we could use it

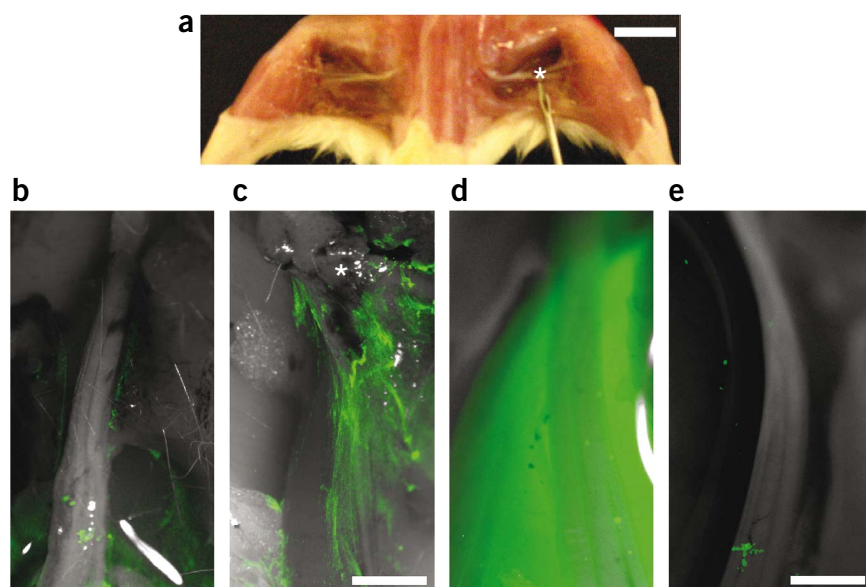


Figure 4 | *In vivo* imaging of axonal degeneration using pSIVA_m. (a) Photograph of exposed sciatic nerves of a rat before transection on the right side. The sciatic nerve was transected just below the greater trochanter, indicated by an asterisk. (b,c) Overlay of fluorescence and brightfield images of the untransected (b) and the transected (c) sciatic nerve. pSIVA_m was applied by an intramuscular injection along the sciatic nerve before imaging. Asterisk marks the site of the injury. (d,e) Overlay of fluorescence and brightfield images of an uninjured sciatic nerve stained with FITC-labeled anxA5 (d) or pSIVA_m (e). pSIVA_m and FITC-labeled anxA5 were applied directly on the exposed nerves. Scale bars, 1 cm (a) and 3 mm (c,e).

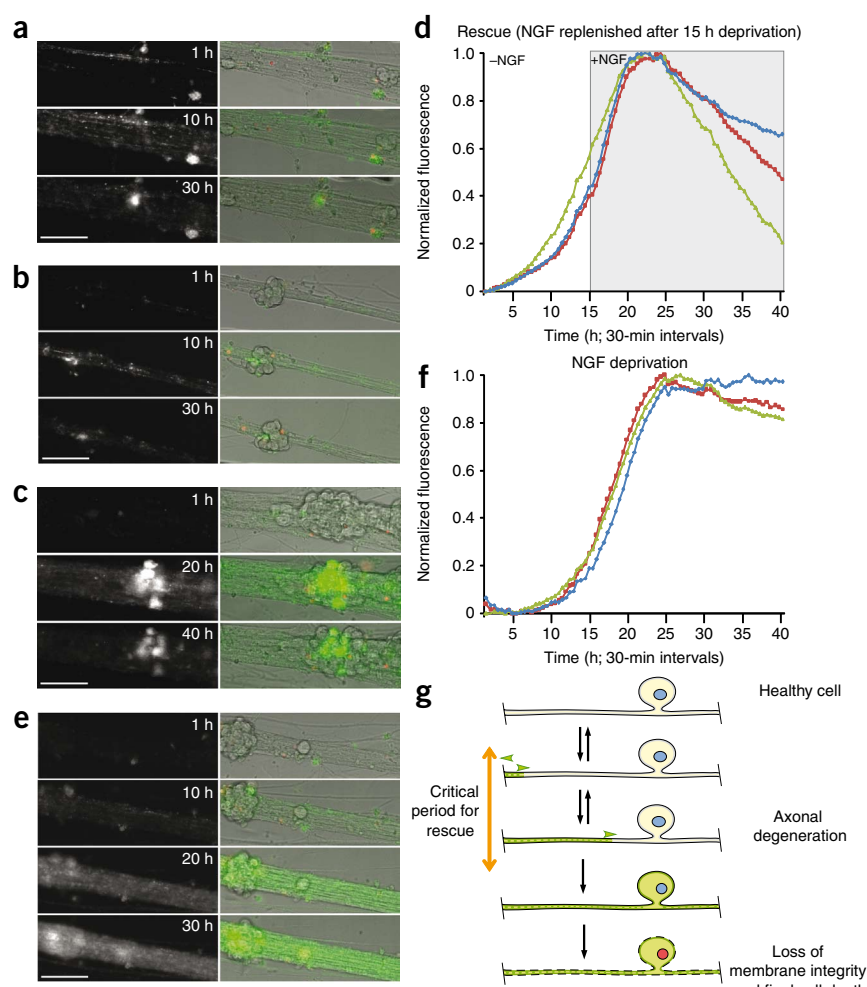


Figure 5 | Rescue of neuronal degeneration visualized by pSIVA_m. (**a–f**) DRG neurons dependent on trophic support were induced to undergo apoptosis by deprivation of NGF for 7 h (**a**), 10 h (**b**) or 15 h (**c**) before NGF was replenished in the culture medium and under continuous NGF deprivation (positive control; **e**). pSIVA_m fluorescence (left) and merged phase contrast, green (pSIVA_m) and red (PI) fluorescence (right) images at indicated times after initial NGF removal are shown. Scale bars, 100 μm. Total fluorescence measured in three fields of view (indicated by different colors) for pSIVA_m in the presence of NGF-deprived neurons (**d**) and in the presence of neurons that had been deprived of NGF for 15 h before induction of rescue by the re-addition of NGF to the culture medium (**f**). Cells were imaged with pSIVA_m and PI present in the culture medium for the duration of the experiment. (**g**) Summary of the progression and/or recovery of early apoptotic events in the neuron as detected by pSIVA_m.

localized cellular processes involved in the initial stages of axonal degeneration^{18,19}. The punctate staining pattern of pSIVA_m in the axons was characteristic of binding to the PS exposed on the outer leaflet of the plasma membrane rather than binding to the PS from the intracellular side where PS is more abundant and uniformly distributed (**Supplementary Fig. 3**). We observed the loss of membrane integrity and cell death, indicated by PI incorporation (**Fig. 3c** and **Supplementary Videos 1,2**) typically ~1 h after PS exposure on the cell bodies.

The advantages of pSIVA also extend to imaging apoptotic processes *in vivo*. To test the utility of pSIVA for *in vivo* applications, we administered the biosensor by an intramuscular injection of pSIVA_m along the rat sciatic nerve 3 d after nerve transection and imaged degenerating neurons (**Fig. 4**). Except for some minor staining of tissue damaged while exposing the sciatic nerve for imaging, pSIVA_m exclusively stained axons in the sciatic nerve distal to the site of injury²⁰ (**Fig. 4c**), whereas staining in the contralateral control (uninjured) sciatic nerve was undetectable (**Fig. 4b**). We observed punctate staining in the sciatic nerve axons, reminiscent of the staining observed in degenerating axons of DRG neurons *in vitro* (**Fig. 3**).

To compare pSIVA_m and conventional annexin dyes in *in vivo* and live imaging applications, we applied 10 μg of FITC-labeled anxA5 to the exposed sciatic nerve on one side of an uninjured rat (**Fig. 4d**) and 100 μg of pSIVA_m to the uninjured nerve on the contralateral side (**Fig. 4e**). There was a notable difference in the background signal from pSIVA_m and enhanced signal-to-noise ratio of pSIVA_m fluorescence.

Rescue of neuronal degeneration

Previous reports have indicated that the initiation of the apoptotic program is not necessarily indicative of a terminal commitment to cell death^{21–23}. In neurons, axonal degeneration is a highly regulated and dynamic process, which involves apoptotic mechanisms without necessarily resulting in cell death²⁰. Understanding the temporal

to analyze a more complex process, such as neuronal degeneration. Under different conditions, axonal degeneration and death can occur at different times and independently from each other¹⁷. We used pSIVA_m to study axonal degeneration using purified DRG sensory neurons, which have a single axon without dendrites.

Because DRG neurons are dependent on trophic factor support for survival, we induced degeneration by deprivation of NGF and monitored the process via time-lapse microscopy. Similar to what we observed in COS-7 cells, we observed a lag of several hours between initial PS exposures in the axons and complete cell death, determined by PI staining of the nuclei (**Supplementary Videos 1** and **2**). We observed pSIVA_m staining in both axons and cell bodies of NGF-deprived neurons whereas pSIVA_m fluorescence was largely absent in neurons grown in the presence of NGF (**Fig. 3a,b**). We observed a gradual increase in fluorescence intensity in the NGF-deprived neurons, corresponding to both a gradual increase in amount of PS exposure in an individual neuron and also the number of degenerating neurons present over longer periods of NGF deprivation (**Fig. 3b** and **Supplementary Videos 1,2**). Furthermore, pSIVA_m binding occurred in a specific spatiotemporal order, indicating that PS exposure occurred successively, originating from a particular location in the axon and progressing toward the cell body or the axon terminal (**Fig. 3c,d** and **Supplementary Videos 1,2**). Inspection of PS exposure on a single axon revealed a dynamic, sequential punctate staining pattern (**Fig. 3c,d**), which may be indicative of the underlying

dynamics of whether and when rescue is possible in degenerating neurons will provide an indication of the severity of the cellular response and the time window in which neuronal survival mechanisms can still be effective. Although these studies have been difficult to perform in the past, using time-lapse microscopy and pSIVA_m, we set out to define the critical period when apoptotic processes are reversible.

We first initiated degeneration in DRG neurons by NGF deprivation and then added back NGF once we detected PS exposure on the axons (at 7, 10 and 15 h after initial NGF removal). To quantify this process, we measured the total fluorescence for multiple fields of view occupied by axons. For neurons that were deprived of NGF, we measured low fluorescence intensity for the first 13–15 h, followed by a period of increasing fluorescence intensity, indicative of the amount of PS exposure (**Supplementary Fig. 1k**). We blocked complete cell death by re-administering NGF in some cells but not all. Concurrent with neuronal survival, we observed a decrease in pSIVA_m fluorescence, indicating that binding decreased as PS was restored to the inner leaflet of the plasma membrane (**Fig. 5a–c** and **Supplementary Videos 3,4**). For neurons that were deprived of NGF for 15 h before the initiation of rescue by replenishing NGF to the culture medium, maximum fluorescence intensities were reached at 20–24 h, followed by a period of decreasing fluorescence intensity, which continued to the end of the 40-h time course (**Fig. 5d**). The variation in the total fluorescence intensity measured for different fields after the induction of rescue (**Fig. 5d**) likely represents the different vulnerabilities and timings of the neurons in each particular field. The fluorescence intensities measured for neurons grown under normal conditions remained low throughout the 40-h experiment (data not shown). In comparison, when the neurons were continuously deprived of NGF, pSIVA_m fluorescence increased as before but was then maintained to the end of the experiment (**Fig. 5e,f** and **Supplementary Videos 1,2**).

We observed rescue from degeneration in neurons with pSIVA_m-stained axons but not in neurons with pSIVA_m-stained cell bodies (**Fig. 5a–c**), which was indicative of later stages of cell death (**Fig. 3** and **Supplementary Videos 1–4**). Therefore, we deduced that rescue was observed in neurons that were still in early stages of degeneration (**Fig. 5g**). Some axons retained pSIVA_m staining after the re-administration of NGF, indicating that in intermediate stages, rescue was not possible. We observed the rescue of single neurons from apoptosis when NGF was re-administered within 7–15 h after initial NGF withdrawal. This relatively large time may be attributed to the heterogeneous timing of the responses owing to the different vulnerabilities of individual neurons, in addition to the critical period after the initiation of apoptotic mechanisms in which rescue is still possible (after PS exposure in the axon but before PS exposure in the cell body).

DISCUSSION

The main technical advance of this approach is in the design of pSIVA, which couples binding to PS-containing membranes directly to polarity-sensitive fluorescence emission. This makes pSIVA an ideal tool for live-cell imaging because it can be used in excess and its continuous presence in the cell culture medium is undetectable until PS binding occurs. Therefore, pSIVA allows for high-sensitivity and instantaneous visualization of PS exposure on cells.

The sequential punctate pSIVA_m staining we observed at various localized areas of PS-exposure in purified neuronal cultures may be an indication of the movement of localized apoptotic signaling processes through the neuron^{18,19}. Recently, caspase 6 localization has been observed in a similar punctate staining pattern²⁴. The similarities in the appearance of punctate PS exposure and punctate caspase 6 activation in axonal degeneration supports pSIVA as an indicator to visualize degeneration occurring in real time at localized areas in axons. Our observation of localized pSIVA_m staining in both DRG neurons and COS-7 cells undergoing cell death, suggests that heterogeneity in PS exposure on the plasma membrane is a general occurrence. However, additional studies are required to characterize the underlying cellular mechanisms.

pSIVA_m has several advantages over previous annexin-based probes because we optimized it for live-cell and *in vivo* imaging methods. The ability to immediately visualize local membrane changes in the initial stages of apoptosis is important because it provides an indication of where the first cellular events are occurring. pSIVA_m provides the means to investigate the time course of apoptotic mechanisms at the single cell level, regardless of cell-to-cell variations in the response of neighboring cells to the same environment. Furthermore, in combination with current and future technical advances in *in vivo* imaging methods, we anticipate a utility of pSIVA_m for examining the spatiotemporal progression of cell death and degeneration in model systems and after injury or disease. As pSIVA_m binding is reversible, this sensor is particularly suitable for the study of apoptotic processes in which progression into cell death is not obligatory, such as in Wallerian degeneration, axonal pruning and axonal degeneration. Combined with other cellular markers, pSIVA_m may be used to directly investigate how signals for survival might counteract the apoptotic pathway in disease, development and cell homeostasis.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemethods/>.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank the members of the Chan lab for technical assistance and M. Cayouette for commenting on the manuscript. This work was partially supported by the US National Multiple Sclerosis Society Career Transition Award (J.R.C.), EY12155 (J.C.). J.R.C. is a Harry Weaver Neuroscience Scholar (TA 3008A/T, JF 2142-A-2).

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. wrote the paper.

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ONLINE METHODS

Protein purification and labeling. Cysteine mutations were made in the appropriate sites in cysteine-less variants of anxA5 (C316A) and anxB12 (C113A,C302A)²⁶ plasmids by site-directed mutagenesis (QuickChange; Stratagene) and expressed and purified as described previously^{14,26}. The anxB12 cysteine-less variant and anxB12 Cys260 had been described previously¹⁴, and oligos used to make anxA5 Cys262, anxB12 Cys4, anxB12 Cys101, and anxB12 Cys101,Cys260 are listed in **Supplementary Table 1**.

The proteins were reacted with a tenfold molar excess of IANBD or BADAN at the introduced cysteine sites (~2 h at room temperature (25 °C) or overnight at 4 °C). Labeling was quenched with a twofold molar excess of β -mercaptoethanol, and labeled proteins were eluted with a PD-10 column in 20 mM Hepes buffer containing 100 mM NaCl at pH 7.4 (Hepes-NaCl).

In vitro membrane binding assay. Large unilamellar vesicles (LUVs) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or a 1:3 molar ratio of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-(phospho-L-serine) (POPS):POPC (Avanti Polar Lipids) were prepared as described previously²⁷. To induce binding, 1 μ M annexin protein was mixed with 1 mM lipid in Hepes-NaCl buffer containing 1 mM Ca^{2+} . The fluorescence emission was measured using a 1 cm path-length quartz cuvette in a Jasco FP-6500 spectrofluorometer. The excitation wavelength for IANBD was set to 478 nm, and fluorescence emission was monitored from 480 to 650 nm. For BADAN, the excitation wavelength was set to 380 nm, and fluorescence emission was monitored from 400 to 650 nm. For the comparison of pSIVA_m to AnxV-FITC, 1 μ M annexin protein was incubated with lipid vesicles at various molar ratios (1:1,000, 1:500, 1:250, 1:100, 1:50, 1:10 and 1:1) and the fluorescence emission intensity was measured at the different protein:lipid ratios for anxB12 Cys101, Cys260-IANBD and compared with AnxV-FITC (excitation maximum = 485 nm, emission maximum = 520 nm).

COS-7 cell culture. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin and grown on tissue culture plates 24–48 h before live imaging experiments. Apoptosis was induced in COS-7 cells by 100 μ M etoposide, and these cells were compared with healthy cells grown without the addition of etoposide (DMSO only).

Primary neuron cultures. DRG neurons were isolated and purified as previously described²⁸. Briefly DRG neurons from embryonic day (E)13–E15 Sprague-Dawley rats were dissociated, plated and purified on collagen-coated tissue culture plates in the presence of NGF (100 ng ml⁻¹) for 7 d before imaging. Neurons were grown in etched wells to direct the orientation of axon growth along a single axis. Apoptosis was induced by removal of NGF from the culture medium. For rescue of neuronal degeneration, NGF was replenished (100 ng ml⁻¹) after 7, 10 and 15 h of deprivation.

Time-lapse microscopy and live-cell imaging. Time-lapse microscopy was performed on an Axiovert 200 motorized inverted

microscope equipped with a complete incubation system (Zeiss). Time-lapse images of COS-7 cells were taken with the AxioCam MRm digital camera (Zeiss). To minimize phototoxicity, neurons were imaged with a Cascade: 1K camera (Photometrics), which reduced exposure times. All images were processed using Axiovision 4.7 software (Zeiss). Cells were imaged in the presence of pSIVA_m (5–10 μ g ml⁻¹) and PI (1 μ M).

Cosedimentation assay. We incubated 5 μ g of the various IANBD- or BADAN-labeled annexin proteins at a 1:1,000 molar ratio with sucrose-filled large multilamellar vesicles composed of 100% POPC or 25% POPS–75% POPC (as described previously¹²) in Hepes-NaCl buffer containing 1 mM Ca^{2+} to induce binding. The mixture was centrifuged for 10 min in a tabletop centrifuge (15,000g, 4 °C). The supernatant was concentrated with 10,000 Da molecular weight cut-off (MWCO) centrifugal filters before analyzing by SDS-PAGE as the solution fraction. The lipid pellets were incubated in 10 μ l of buffer containing 3 mM EDTA to reverse the binding of the annexin proteins to membranes. The mixture was centrifuged and the supernatant was analyzed by SDS-PAGE as the lipid pellet fraction.

Immunostaining. Immunostaining of DRG neuron cultures was performed as previously described²⁹. Briefly, cultures were deprived of NGF for 20 h to induce degeneration. Cultures were then fixed in 4% paraformaldehyde and then permeabilized and blocked by incubation with 20% goat serum and 0.2% Triton X-100 in PBS (pH 7.4). Cells were then washed and incubated for 1 h with pSIVA_m and the neurofilament antibody (ATCC) before washing and application of the secondary antibody for neurofilament. For live-cell staining, DRG neurons were deprived of NGF for 20 h and then subsequently incubated with pSIVA_m and neurofilament antibody (ATCC) in D-PBS for 15 min, before fixation in 4% cold paraformaldehyde (10 min). The cells were permeabilized in 0.02% digitonin (30 min) before blocking and staining with the secondary antibody.

In vivo transection and imaging of rat sciatic nerve. Sprague Dawley rats (one month postnatal) were anaesthetized with an intraperitoneal injection of a premixed solution containing ketamine (50 mg kg⁻¹) plus xylazine (5 mg kg⁻¹). After shaving and preparing the skin, the sciatic nerve from one side of the rat was exposed and transected just below the greater trochanter using surgical Moria spring scissors with a 5 mm blade (Fine Science Tools). The contralateral sciatic nerve was exposed but was not transected. The wound was closed with 50 Vicryl (Ethicon) in the muscle fascia and 50 Dermalon (Ethicon) sutures in the skin. The rats were given subcutaneous injections of buprenex-buprenorphine at 0.05 mg kg⁻¹ daily and housed in standard cages and fed laboratory chow and water. Three days after transection, pSIVA_m was applied by an intramuscular injection below the greater trochanter on the left (uninjured control) and right (injured) sides of the animal. The nerves were exposed for imaging. Animal care and experimental procedures were monitored by the investigators and approved by the University of Southern California Institutional Animal Care and Use Committee (Animal Protocol number 11182).

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