Chapter 4

Detection of Initiator Caspase Induced Proximity in Single Cells by Caspase Bimolecular Fluorescence Complementation

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Abstract

The caspase family of proteases includes key regulators of apoptosis and inflammation. The caspases can be divided into two groups, the initiator caspases and the executioner caspases. Initiator caspases include caspase-2, caspase-8, and caspase-9 and are activated by proximity-induced dimerization upon recruitment to large molecular weight protein complexes called activation platforms. This protocol describes an imaging-based technique called caspase *Bi*molecular *F*luorescence *C*omplementation (BiFC) that measures induced proximity of initiator caspases. This method uses nonfluorescent fragments of the fluorescent protein Venus fused to initiator caspase monomers. When the caspase is recruited to its activation platform, the resulting induced proximity of the caspase monomers facilitates refolding of the Venus fragments into the full molecule, reconstituting its fluorescence. Thus, the assembly of initiator caspase activation platforms can be followed in single cells in real time. Induced proximity is the most apical step in the activation of initiator caspases, and therefore, caspase BiFC is a robust and specific method to measure initiator caspase activation.

Key words Apoptosis, BiFC, Caspases, Confocal, Fluorescence, Imaging, Microscopy, Venus

1 Introduction

The caspases are a family of *c*ysteine-dependent *asp*artate-directed prote*ases* that are responsible for the initiation and execution of apoptosis. The caspases are classified as either initiator caspases, including caspase-2, -8, -9, and -10, or executioner caspases, such as caspase-3, -6, and -7 [1]. Initiator caspases are so-called because they are the first to be activated in a pathway. The predominant function of active initiator caspases in apoptotic pathways is to activate downstream executioner caspases. Active executioner caspases then proteolytically cleave numerous regulatory and structural proteins within the cell usually via cleavage at specific aspartate residues [2]. A third group of caspases exists that includes caspase-1, -4, -5, -11, and -12. These caspases are similar in structure to

Hamsa Puthalakath and Christine J. Hawkins (eds.), *Programmed Cell Death: Methods and Protocols*, Methods in Molecular Biology, vol. 1419, DOI 10.1007/978-1-4939-3581-9_4,

the initiator caspases but are not involved in the regulation of apoptosis. Members of this group are referred to as inflammatory caspases and have key roles in the regulation of innate immunity.

Initiator caspases are activated by proximity-induced dimerization following recruitment to large molecular weight complexes known as activation platforms (Fig. 1). For example, ligation of death receptors such as TNFR1, TRAIL, or Fas by their cognate ligands leads to the formation of the activation platform for the initiator caspase-8. This activation platform is a multimeric signaling complex called the DISC (*d*eath-*i*nducing *s*ignaling *c*omplex) [3]. The DISC consists of proteins that oligomerize via homotypic protein–protein interaction domains. The main component linking death receptor ligation to caspase-8 activation is a protein called FADD [4]. FADD contains two protein–protein interaction motifs: a *d*eath *d*omain (DD) and a *d*eath *e*ffector *d*omain (DED) [5]. FADD binds to the intracellular portion of the oligomerized death receptor via DD interactions in FADD and the death receptor (e.g., Fas). FADD then recruits caspase-8 molecules via DEDs in both proteins.

Binding of caspase-8 molecules in the DISC brings them into close physical proximity in a process known as induced proximity [6, 7]. Induced proximity facilitates dimerization of caspase-8 molecules, at which point the caspase is active. Once dimerized, caspase-8 is auto-processed, that is, the catalytic domain of one caspase-8 molecule mediates cleavage of the other caspase-8 molecule between the large and small subunit. Further processing removes the prodomain generating a heterodimer comprised of the large and small catalytic subunits [8]. This cleavage is required to stabilize the active caspase-8 dimer [9].

Caspase-9 is similarly activated by induced proximity. Release of cytochrome c from mitochondria following *m*itochondrial *o*uter *m*embrane *p*ermeabilization (MOMP) induces the assembly of a complex known as the apoptosome (Fig. 1), which comprises cytochrome c, dATP, and APAF-1. The apoptosome functions as an activation platform for caspase-9, recruiting caspase-9 molecules via binding between protein–protein interaction motifs called CARDs (*ca*spase-*r*ecruitment *d*omains) that are present in the prodomain of caspase-9 and in the APAF-1 sequence [10].

Caspase-2 appears to be activated in an activation platform termed the PIDDosome (Fig. 1), which facilitates induced proximity and dimerization of caspase-2 monomers [11]. The PIDDosome consists of the PIDD and the adaptor protein RAIDD that interact via the DD in both their sequences. RAIDD, in turn, recruits caspase-2, and this binding is mediated by the CARD present in both proteins. Certain stimuli, such as heat shock and cytoskeletal disruption, have been shown to activate caspase-2 [12, 13], but it is unclear if such stimuli are dependent on PIDDosome assembly or if alternative caspase-2 activation platforms exist [14].

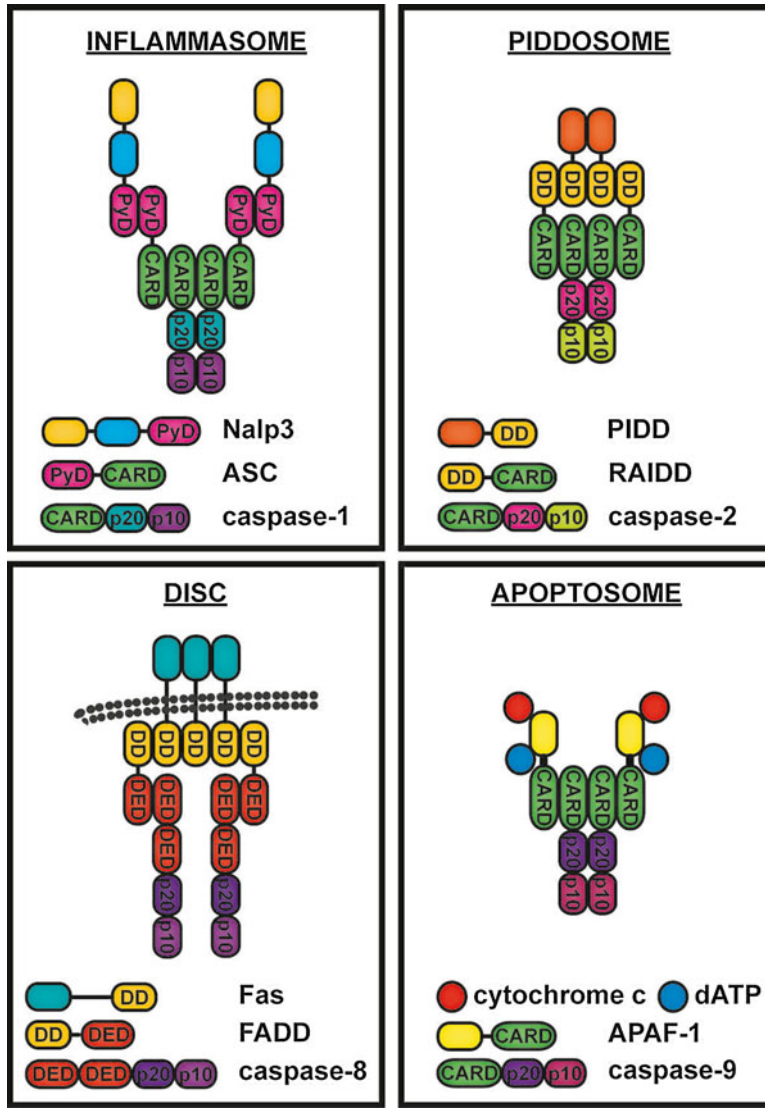

Fig. 1 Initiator caspases are activated by induced proximity upon recruitment to specific activation platforms. Examples of activation platforms that specifically recruit caspase-1, caspase-2, caspase-8, or caspase-9 are depicted. Caspase-1 is activated by inflammasomes, such as the Nalp3 inflammasome that is shown here. Proinflammatory stimuli, such as peptidoglycan, induce aggregation of Nalp3, which enables recruitment and clustering of the adaptor protein ASC. ASC, in turn, recruits caspase-1 monomers to the complex. Caspase-2 is similarly recruited to the PIDDosome comprising of PIDD and the adaptor protein RAIDD. Engagement of death receptors at the cell membrane, such as Fas, by their cognate ligands, such as FasL, results in assembly of the DISC that recruits caspase-8. The APAF-1 apoptosome is assembled in response to cytochrome c release from the mitochondria. Cytochrome c and dATP bind to APAF-1 leading to the assembly of the apoptosome and recruitment of caspase-9 monomers. The assembly of each of these activation platforms is governed by protein–protein interactions mediated by conserved protein binding motifs including the *ca*spase *r*ecruitment *d*omain (CARD), the *d*eath *d*omain (DD), the *d*eath *e*ffector *d*omain (DED), and the *py*rin *d*omain (PyD). Recruitment of initiator caspase monomers to their specific activation platform results in induced proximity, dimerization, and activation of each caspase

The inflammatory caspases are not primarily involved in apoptosis but they are similarly activated upon recruitment to activation platforms. Caspase-1 is activated by dimerization following recruitment to the inflammasome (Ref. [15] and Fig. 1). At least four distinct inflammasomes exist and each one appears to be assembled in response to specific pathogen products such as bacterial cell wall components or viral DNA [16]. Upon activation, caspase-1 cleaves the proinflammatory cytokines pro-interleukin-1β and pro-interleukin-18 to their mature forms and has fundamental roles on a variety of aspects of innate immune function [17].

Induced proximity is the most apical step in the activation of each initiator caspase. Caspase *Bi*molecular *F*luorescence *C*omplementation (BiFC) was developed to allow direct visualization this step following recruitment to activation platforms in real time, in single cells. BiFC is a method that uses split fluorescent proteins to measure protein–protein interactions in cells, and was first described for monitoring the interaction between the proteins Fos and Jun in live cells [18]. BiFC employs a fluorescent protein such as Venus (a brighter and more photostable version of *y*ellow *f*luorescent *p*rotein (YFP)) that is split into two fragments that are not fluorescent by themselves. When these protein fragments are fused to proteins that interact, the Venus fragments associate to reform the fluorescent molecule. Experimentally, this is detected by visualizing Venus fluorescence in live cells.

Caspase BiFC adapts this technique to measure the recruitment of initiator caspases to activation platforms (Fig. 2). Caspase BiFC was originally developed to interrogate the caspase-2 pathway [19] and has more recently been adapted to measure induced proximity of the inflammatory caspases -1, -4, -5, and -12 [20]. To achieve this, either the entire caspase or the portion of the caspase that binds to the activation platform (i.e., the prodomain that contains a CARD or DEDs) is fused to each fragment of Venus (Fig. 2a). In untreated cells, the caspase monomers maintain the Venus fragments separated from each other. The caspase-BiFC fusion proteins are recruited to the activation platform in response to an activating stimulus, such as vincristine for caspase-2 [19]. The resulting induced proximity of caspase BiFC fusion proteins promotes association of the Venus fragments, thereby restoring fluorescence (Fig. 2b, c). Thus, Venus fluorescence can be used as a measure of initiator caspase activation (Fig. 2d).

Caspase BiFC measures induced proximity as opposed to measuring cleavage of the caspase, which is a more downstream event. Executioner caspases are activated by cleavage and therefore monitoring disappearance of the full-length protein and the concurrent appearance of its cleavage products by western blot is a bona fide measure of activation [21]. However, initiator caspases are activated by dimerization and subsequent auto-processing stabilizes the active enzyme [21]. Importantly, cleavage of initiator caspases

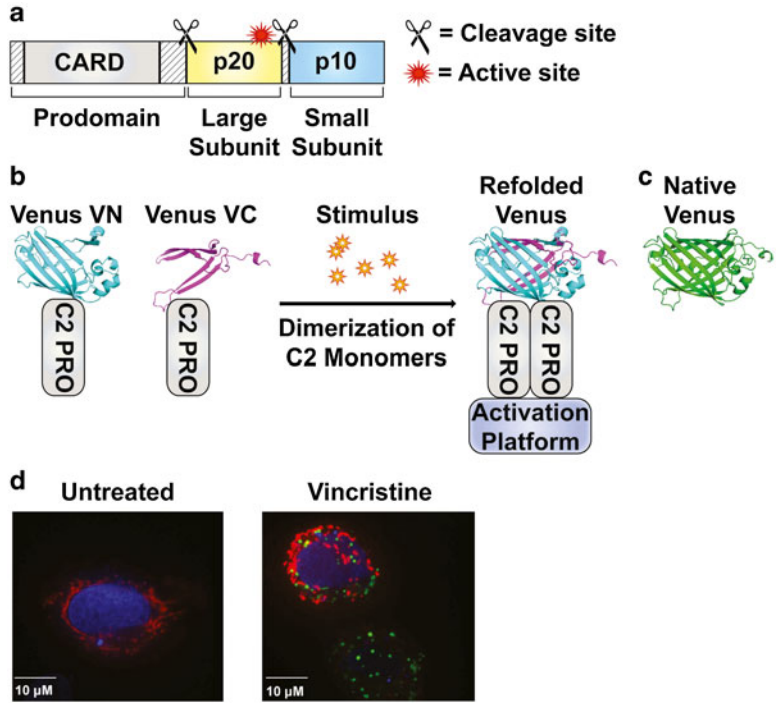

Fig. 2 Caspase Bimolecular Fluorescence Complementation measures induced proximity of initiator caspases. (**a**) Each initiator caspase is comprised of a large catalytic subunit (p20), a small catalytic subunit (p10), and a prodomain that contains one or two protein binding motifs; either a CARD, as illustrated, or two DEDs,. (**b**) Caspase BiFC fuses the prodomain of an initiator caspase to the N-terminus of Venus (Venus VN) or to the C-terminus of Venus (Venus C). Caspase-2 is shown here as an example. The initiator caspase-BiFC fusion proteins remain as monomers in unstimulated cells and the two halves of Venus do not refold. Following a caspase activating stimulus, the caspase fusion protein monomers are recruited to the activation platform. This allows for the two halves of Venus to be in sufficient proximity so that they refold and reconstitute the full length Venus protein, leading to fluorescence. (**c**) The protein structure of native Venus is shown for comparison. The molecular models of the protein structures were created using PyMOL v1.7.4.4 Edu with the amino acid sequences for Venus N (amino acids 1–173), Venus C (amino acids 155–238) and full length Venus. (**d**) The images show an example of caspase-2 BiFC in live cells induced by the cytoskeletal disruptor, vincristine. The caspase-2 Pro-BiFC components were expressed in Hela cells and the images were acquired using a spinning disk confocal microscope. The green fluorescence in the cells treated with vincristine (*right panel*) represents caspase-2 BiFC and is not detected in the untreated cells (*left panel*). Mitochondria are shown in red and represent expression of the reporter gene, DsRed-Mito. Nuclei were stained with Draq5 and are shown in *blue*

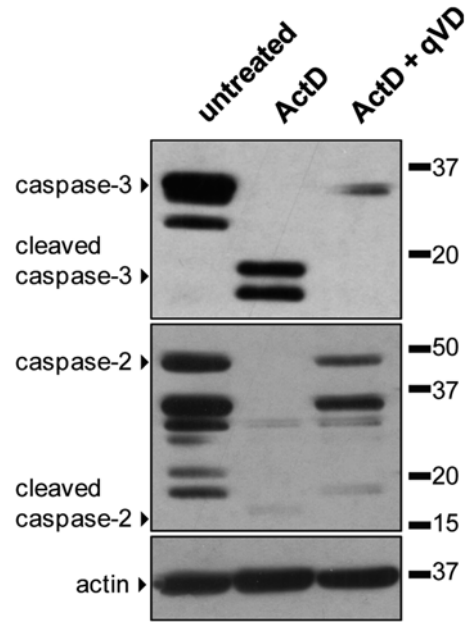

Fig. 3 Measuring caspase cleavage by western blot is not sufficient to determine activation of initiator caspases. In the example shown, *Act*inomycin *D* (ActD) induces cleavage of the executioner caspase, caspase-3, as measured by disappearance of the full length protein accompanied by appearance of the cleaved subunits. The initiator caspase, caspase-2, was also cleaved in the cells treated with ActD. However, ActD does not activate caspase-2 and this cleavage is likely mediated by caspase-3 rather than representing caspase-2 activation

in the absence of dimerization is not sufficient for activation [8, 22, 23]. In the example shown in Fig. 3, caspase-3 cleavage in lysates of cells treated with *Act*inomycin *D* (Act D) is easily detected. Caspase-2 cleavage is also detected in response to Act D. However, it has been shown that Act D does not activate caspase-2 [19, 24]. Therefore, this cleavage is most likely mediated by caspase-3 rather than representing the auto-processing step that is associated with activation of the initiator caspase. Single cell imaging of caspase BiFC, in contrast, allows for specific analysis of initiator caspase interactions at the level of the activation platform. This can provide a precise determination of the number of caspase BiFC events in a cell population, the subcellular location of caspase activation, and the kinetics of initiator caspase activation.

The method described here outlines how to introduce the caspase BiFC components into cells and how to visualize caspase BiFC in response to pro-apoptotic stimuli. Different approaches to acquiring and analyzing the data are detailed. These include determination of the percentage of caspase induced proximity at a single time point and visualization of activation platform assembly in single cells in real time using high resolution time-lapse confocal microscopy.

2 Materials

2.1 Plasmids

1. Plasmid constructs encoding the full length catalytically inactive caspase or the prodomain of the caspase fused to either the N-terminus of Venus (VN) or the C-terminus of Venus (VC):
   (a) BiFC plasmids for caspase-1, caspase-2, caspase-4, caspase-5, and caspase-12 were described in [19, 20]. These plasmids are available by request from the authors.
   (b) The BiFC plasmids, pBiFC.VC155 and pBiFC.VN173, are available from Addgene (#22011 and #22010). Additional caspases can be cloned into these plasmids by standard molecular cloning techniques.
2. Control plasmids: Plasmids containing point mutations that disrupt the binding interface between the caspase and its activation platform. Binding mutants for caspase-2 and caspase-1 were described in [19, 20] and are available by request from the authors.
3. Fluorescent reporter plasmid: A red or cyan fluorescent plasmid to be used as a reporter for transfection. This protocol uses DsRed-Mito (Clontech).

2.2 Cell Culture and Transfection

1. Cells can be obtained from the appropriate culture collections such as ATCC. HeLa cells were used in this protocol (ATCC).
2. Cell growth medium: Media and supplements should be chosen that are appropriate for each cell line. Hela cells are cultured in standard *D*ulbecco's *M*odified *E*agle *M*edia (DMEM), supplemented with *f*etal *b*ovine *s*erum (FBS, 10 % (v/v)), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL).
3. Imaging medium: Cell growth medium supplemented with Hepes (20 mM, pH 7.2–7.5) and 2-mercaptoethanol (55 μM).
4. *P*hosphate *b*uffered *s*aline (PBS).
5. Trypsin–EDTA.
6. Serum-free growth medium: This protocol uses Opti-MEM (Life Technologies).
7. Transfection reagent: This protocol uses Lipofectamine 2000 reagent (Life Technologies).
8. Glass bottom 3.5 cm dishes or 6-well plates, number 1.5 thickness (0.16–0.19 mm) (Mattek Corporation).
9. Fibronectin solution (1 mg/mL): Dilute in PBS to a final concentration of 0.1 mg/mL and store at 4 °C.

2.3 Induction of Apoptosis

1. qVD-OPh: resuspend in DMSO at 20 mM, store in aliquots at -20 °C, avoid repeated freeze/thaw.
2. Stimuli to induce apoptosis or caspase activation: Common inducers of apoptosis are listed below. All reagents listed should be stored in aliquots at –20 °C and repeated freeze–thaw should be avoided:
   (a) Actinomycin D: resuspend in DMSO at 1 mM.
   (b) TNFα: resuspend in sterile distilled water at 0.1 mg/mL.
   (c) Cycloheximide: resuspend in ethanol at 10 mg/mL.
   (d) Vincristine: resuspend in sterile distilled water at 20 mM.

2.4 Microscope Instrumentation

1. Microscope: In the protocol below we use a spinning disk confocal microscope for time-lapse or high resolution imaging, and an epi-fluorescence microscope for single time point analysis. Some useful microscope options include:
   (a) Motorized stage.
   (b) A heated stage or enclosed incubator to control the temperature and CO_2 concentration.
   (c) A camera to detect and image the cells: A CCD (*c*harge *c*oupled *d*evice) or EMCCD (*e*lectron *m*ultiplying *CCD*) camera offers high resolution and fast imaging.
   (d) Focal drift correction system such as Definite Focus (Zeiss).
2. Laser source: Venus is optimally excited by a 514 nm laser line (*see* **Note 1**). A second laser line is required to excite the fluorescent reporter (e.g., 458 nm for CFP or 561 nm for RFP).
3. Epi-fluoresence illumination:
   (a) Fluorescence filter sets consisting of an excitation filter, a dichroic beamsplitter, and an emission filter for the YFP or GFP wavelengths.
   (b) Fluorescence filter set to excite the fluorescent reporter such as RFP.

2.5 Data Processing

1. Microscope acquisition software.
2. Computer to run the microscope acquisition software: The computer should have a modern processor. A 64 bit computer is recommended for many types of acquisition software.
3. Image analysis software: This protocol uses ImageJ (NIH).
4. Graphing software such as Microsoft Excel.

3 Methods

3.1 Transfection of Caspase BiFC Plasmids

1. One day prior to transfection, coat 3.5 cm glass bottom dishes or a 6-well plate with embedded coverslips with fibronectin (0.1 mg/mL) (*see* **Note 2**). Add 1 mL per dish and incubate for at least 5 min at room temperature. Remove the fibronectin, wash wells once in PBS and remove PBS (*see* **Note 3**).
2. Plate 1×10^5 Hela cells per dish or well of a 6-well plate. Scale down as appropriate for smaller plate surface areas (*see* **Notes 4** and **5**).
3. The following day, add 12 μL Lipofectamine 2000 to 750 μL Opti-MEM in a sterile tube for 6×3.5 cm dishes or a 6-well plate and scale up or down as needed (*see* **Note 6**).
4. Incubate at room temperature for 5 min.
5. Add the appropriate amounts of BiFC plasmids and fluorescent reporter plasmid to a separate sterile microcentrifuge tube in a total of 100 μL Opti-MEM for each dish or well of a 6-well plate (*see* **Notes 7** and **8**).
6. Add 100 μL of the Lipofectamine solution from **step 3** to each plasmid solution.
7. Incubate at room temperature for 20 min to allow the DNA-lipid complexes to form.
8. Aspirate the growth medium from the cells and add 800 μL of serum free DMEM, pre-warmed to 37 °C, to each dish or well.
9. Add the DNA-lipid complexes (200 μL total) to each dish or well in a dropwise manner, followed by gently swirling of the dish to assure equal distribution throughout.
10. After 3 h, exchange the media with complete growth media, pre-warmed to 37 °C (*see* **Note 9**).
11. Incubate cells at 37 °C for at least 24 h to allow expression of the caspase BiFC components.

3.2 Induction of Apoptosis

1. Treat cells with the chosen death stimulus (*see* **Note 10**).
   (a) For drug treatments: Add the drug to imaging medium at the appropriate concentration and gently mix (*see* **Note 11**). Include a caspase inhibitor such as qVD-OPh (20 μM) if needed (*see* **Note 12**). Add the drug-containing medium to cells.
   (b) For heat shock: Exchange media for imaging media heated to the heat shock temperature. Add qVD-OPh (20 μM) to the heated media if using. Place the plate in an incubator set at the heat shock temperature for the appropriate time (*see* **Note 13**), and then return to 37 °C incubation.

2. Include an untreated control well or plate.
3. Incubate cells for the appropriate amount of time and proceed to Subheading 3.3 or immediately start the time-lapse and proceed to Subheading 3.4.

3.3 Caspase BiFC for Single Time Point Data Acquisition

1. Count the number of cells that are Venus (i.e., BiFC)-positive at the chosen time point using an epi-fluorescence microscope.
2. Visualize the cells under the filter that excites RFP and count all of the DsRed-positive cells in a field.
3. Visualize the same field under the filter that excites GFP and count the number of red cells that are Venus-positive (*see* **Note 14**).
4. Count 3 different areas of the plate, with each area containing at least 100 DsRed-positive cells.
5. Repeat each experiment at least three times to ensure reproducibility.

3.4 Caspase BiFC for Time-Lapse Microscopy

1. Turn on the microscope and set the temperature to 37 °C at least one hour prior to imaging the cells. This will give the microscope enough time to reach thermal equilibrium (*see* **Note 15**).
2. At least 30 min prior to imaging the cells, place the dish on the microscope stage using the correct adaptor.
3. Turn on the CO_2 source (*see* **Note 16**).
4. Locate transfected cells by looking for cells that express the transfection reporter. For example, if using DsRed-Mito, search for cells using the 561 nm laser.
5. Focus on the cells using a 60× or a 63× oil objective (*see* **Note 17**).
6. Determine the lowest amount of laser light and the shortest exposure time required to detect the Venus signal. Note the laser power and exposure times and keep consistent across experiments (*see* **Note 18**).
7. Determine the lowest amount of laser light and shortest exposure times required to detect the signal of the reporter plasmid. Note the laser power and exposure times and keep consistent across experiments.
8. Turn on focus drift correction system if available (*see* **Note 19**)
9. Choose a number of different positions within the plate or across multiple wells (*see* **Note 20**).
10. Set the time interval between each image capture and the duration of the experiment (*see* **Note 21**).
11. Visit each position and fine tune the focus.
12. Begin the time-lapse.
13. At the end of the experiment, save the data.

3.5 Data Analysis

3.5.1 Calculate the Percentage of Venus-Positive Cells at a Single Time Point

1. Using Excel or similar graphing software, enter the number of Venus-positive cells and the total number of transfected cells (e.g., DsRed-Mito-positive cells) for each area of the plate counted and for each experimental condition.
2. Calculate the percentage of Venus-positive cells for each area of the plate and for each experimental condition.
3. Average the percentage of Venus-positive cells of the three (or more) areas per condition, and calculate the standard deviation (*see* **Note 22**).

3.5.2 Calculate the Intensity of Venus-Positive Cells Over Time

1. Use ImageJ or available software to open a series of time-lapse images. Each series is comprised of the images of a single position taken at each time interval (this is called a stack in ImageJ *see* **Note 23**).
2. Using the polygon tool, draw a *r*egion *o*f *i*nterest (ROI) around each DsRed-Mito-positive cell (*see* **Note 24**). In ImageJ, use the ROI manager under "Tools" in the "Analyze" menu. Add the ROIs to the ROI manager by pressing "+" or clicking "Add".
3. Select a small region in an area of the image where there are no cells: this will be the background measurement.
4. Once all ROIs have been generated, save them (*see* **Note 25**).
5. Measure the average intensity of Venus in each region in each frame of the time-lapse. In ImageJ: Go to "Measure" in the ROI manager. A Results window will open up. Open the "Set Measurements" window in the Results window and select "Mean gray value." To record the average intensities of multiple ROIs for the entire stack, select the "Multi Measure" option under "More" in the ROI manager.
6. Export the results to an Excel spreadsheet or similar graphing software.
7. For each cell, subtract the background fluorescence value at each time point.
8. Repeat this process for all the individual positions acquired in the experiment.
9. Average the values for all cells in each treatment group at each time point and graph the results (*see* **Note 26**).
10. Add error bars to each data point by calculating the *s*tandard *e*rror of the *m*ean (SEM) of the cells for each time point.

4 Notes

1. A 488 nm laser can alternatively be used to image Venus, but in this case CFP-based plasmids cannot be used as a reporter for transfection because CFP is also excited at the 488 nm wavelength.

2. Standard plastic tissue culture plates can be used if the epifluorescence microscope is equipped with a long distance objective. Plastic dishes are not suitable for confocal microscopy as they are too thick for the majority of high numerical aperture objectives. In addition, plastic dishes tend to be autofluorescent because they are not sufficiently transparent. Therefore, No. 1.5 glass coverslips that have an average thickness of 0.17 mm are optimal for high resolution confocal imaging.
3. Cells do not adhere to glass surfaces as well as they do to plastic. Coating glass surfaces with fibronectin helps the cell adhere. The fibronectin solution can be reused a number of times. Alternatives to fibronectin include poly-L-lysine or collagen. This step can be omitted if cells are plated on plastic dishes for analysis by epi-fluorescence microscopy.
4. This protocol can be adapted to any adherent cell line. MCF-7 cells and mouse embryonic fibroblasts (MEF) have been used successfully [19, 20]. The transfection reagents and conditions should be optimized for each cell line (*see* **Note 6**).
5. Prepare a sufficient number of plastic or fibronectin-coated dishes or wells to include controls for the experiment such as an untreated control or cells expressing binding mutants of caspases.
6. The protocol outlined here works well for Hela cells, MCF7 cells and MEF, and has been optimized to limit toxicity induced by the transfection reagent. For other cell lines, test different transfection reagents and conditions to find the optimal parameters that give the desired transfection efficiencies with minimal cellular toxicity.
7. 20–40 ng of each caspase BiFC component in a 3.5 cm dish is usually sufficient to transfect Hela or MCF-7 cells. However, if the relative expression is lower, more plasmid should be transfected (100-200 ng). Cells that have lower transfection efficiencies, such as MEF, require higher amounts of plasmid (250–500 ng).
8. A fluorescent reporter plasmid is necessary to label the transfected cells because the cells will not all be Venus-positive. Choose a reporter that is a different and appropriate "color" based on the excitation/emission filters of the microscope that will be used for analysis. For example, if visualizing Venus using a GFP excitation/emission filter instead of a YFP excitation/emission filter on a standard epi-fluorescence microscope, CFP-based plasmids (e.g., CFP, Cerulean, mCyan) should not be used as transfection reporters. This is because CFP is also excited by the GFP filter. In this case, DsRed or a similar RFP-based plasmid (e.g., mCherry, mOrange) is a more suitable

transfection control. DsRed-Mito is used in this protocol and should be transfected at a lower amount than the BiFC plasmids, such as 10 ng per 3.5 cm dish.

9. If plasmids that are known to induce apoptosis are being co-transfected with the BiFC plasmids include a caspase inhibitor such as qVD-OPh (20 μM) in this media. This will prevent cell death induced by the pro-apoptotic protein but will not impact the BiFC (*see* **Note 12**).
10. Some common inducers include: heat shock (1 h at 45 °C for Hela cells) or vincristine (1 μM) to activate caspase-2; TNF plus cycloheximide (10 ng/ml/10 μg/ml) to activate caspase-8; or Actinomycin D (500 nM–1 μM) to activate caspase-9.
11. Imaging media includes Hepes and 2-mercaptoethanol. Hepes is required to buffer the pH. 2-mercaptoethanol prevents accumulation of *r*eactive *o*xygen *s*pecies (ROS) that can be generated by the confocal laser and are toxic to the cells. Phenol red-free media can be used to reduce artifacts due to auto-fluorescence but it is not essential.
12. Adding a pan-caspase inhibitor, such as qVD-OPh, is essential for single time point analysis to prevent the cells detaching from the surface of the dish or plate as they undergo apoptosis. It will not inhibit the BiFC as the probes have no catalytic activity, but will inhibit the execution phase of apoptosis. By inhibiting death, the addition of caspase inhibitors will also limit excessive movement and shape changes of the cells during time-lapse acquisition which greatly facilitates analysis. However, if caspase-dependent downstream effects are being monitored simultaneously, such as MOMP after caspase-2 activation or Annexin V binding, then the caspase inhibitor should be omitted.
13. Cells can alternatively be heat shocked in a water bath. Seal the plates with Parafilm and submerge in water that has been equilibrated to the required temperature.
14. The red cells are cells that have been transfected and are most likely expressing the BiFC plasmids. Cells that are Venus-positive are cells that are positive for BiFC and caspase induced proximity. Occasionally, cells that are not red will still be Venus-positive, but to maintain an objective count, do not include them in your analysis.
15. Cells should be maintained at constant temperature of 37 °C for the duration of the time-lapse experiment to prevent focus drift. Options to help maintain temperature throughout the time-lapse experiment include a temperature controller on the microscope stage, a microscope with an incubator enclosure, or an objective heater.

16. If this option is not available, overlay the media with mineral oil to prevent evaporation of the media from the dish.
17. A 60× or 63× objective is optimal for spinning disk confocal microscopy. However, a 40× objective can be used to capture more cells per field, although there will be a slight reduction in resolution.
18. There will be very little Venus fluorescence at the start of the time-lapse. Therefore, in a separate experiment or by using a positive control within the current experiment, determine the lowest amount of the laser light and exposure time required to image the cells that are BiFC positive. Choose settings where the Venus signal is visible but not saturated. Imaging cells with low levels of laser light and short exposure times will minimize phototoxicity. Control experiments should be carried out either simultaneously or using the same conditions on untreated cells to ensure that the imaging conditions are not phototoxic and that cell division proceeds normally.
19. Focus drift correction will prevent fluctuations in the *z*-plane by maintaining the distance between the objective and the coverslip constant. Thus, changes in focus due to temperature fluctuations can be avoided. Make sure that the correction system can update the *z*-plane for each position chosen. Otherwise the same *z*-plane will be used for all positions.
20. Multiple positions are only possible if the microscope has a motorized XY stage and multi-field capabilities. This allows multiple fields of view to be imaged in the same experiment. Multiple conditions can be assessed side by side if a multi-well chamber slide or if a slide holder that can hold more than one dish is used.
21. These parameters will depend on the timing of caspase activation. There is an increased potential for phototoxicity as the number of images increases. Therefore, if caspase activation or other cellular events following caspase activation is expected in a 16 h window, longer time intervals, such as 5–10 min, are recommended. If caspase activation occurs more rapidly, the time between frames can be reduced. Control experiments in untreated cells should be carried out to determine the maximum number of images that can be taken over a given time period without inducing toxicity or photobleaching.
22. A background level of Venus fluorescence of 10 % or lower in untreated cells is usually acceptable. If the results show a higher background it is likely because the plasmid concentrations are too high, leading to a greater probability of random complementation of the Venus fragments. If this is the case, titrate the amount of the caspase BiFC plasmid pair down to a level where the background is low but the specific signal is still detected.

23. There are different types of software available including Zen (Zeiss), Slidebook (3i), ImageJ (NIH), and Metamorph (Molecular Devices) that can be used for this type of analysis. ImageJ is a freely available and widely used software. Therefore, this protocol outlines specific steps using ImageJ, but other software programs will give identical results.
24. When drawing the ROI, try to minimize black space. It may be difficult to draw a suitable ROI that captures the cell throughout the time-lapse if the cell moves significantly or changes shape. An alternative approach is to draw a large ROI that encompasses all the cell's movements and use segmentation to select the cell within the ROI. Segmentation is a process that separates objects from the background based on differences in intensities and the results are usually represented as a mask. In ImageJ: segment the image by adjusting the threshold. Select "Threshold" under "Adjust" in the "Image" menu. Move the slider until the whole cell is high-lighted and select "Apply" to create the mask. Next "Create Selection" under "Selection" in the "Edit" menu. This will create an ROI that outlines the mask. Add this Selection ROI to the ROI manager. Select the Selection ROI and the ROI that encompasses the cell movements and use the AND function (in the ROI manager, click "More" and then "AND") to create a new ROI that outlines the cell within the chosen region. Measure the intensity of the masked region to follow changes in fluorescence intensity of the single cell throughout the time lapse.
25. In ImageJ, the ROIs are saved as a file. To save, go to the ROI manager, click "More" and then "Save".
26. This analysis generates a read-out for a population of cells. Single cell traces can also be generated, which can be useful for analyzing asynchronous caspase activation events.

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