

A Low-Cost Method for Tracking the Induction of Apoptosis Using FRET-Based Activity Sensors in Suspension Cells

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Abstract

Apoptosis, or programmed cell death, is a tightly regulated cellular event that plays an important role in both normal developmental processes and many pathological states. The induction of apoptosis is tightly regulated through the coordinated action of members of the caspase family of proteases. Here we discuss a relatively inexpensive protocol for monitoring the induction and progression of apoptosis using a genetically encoded fluorescence resonance energy transfer (FRET)-based biosensor of the executioner caspase, caspase-3, in living suspension cells.

Key words Genetically encoded biosensor, Apoptosis, Fluorescence resonance energy transfer, Caspase 3, Sensor for activated caspases based on FRET (SCAT3), Diepoxybutane

1 Introduction

Apoptosis is a form of cell death that plays an important role in both normal physiological processes and disease. Therefore, in order to maintain tissue homeostasis, apoptotic pathways must be properly regulated. Indeed, the dysregulation of apoptotic signaling mechanisms is associated with many pathological states [1–3]. For instance, the upregulation of apoptotic pathways leads to neuronal degeneration associated with dementia while their downregulation is a hallmark of many cancers. In the case of the latter, reduced apoptosis not only promotes tumor formation but it can also adversely affect chemotherapeutic and radiological interventions that induce apoptosis to eliminate cancerous cells [4].

Apoptosis can be divided into two related pathways, the intrinsic and extrinsic pathways (Fig. 1). In both cases, the induction of apoptosis relies on the coordinated activation of cysteinyl, *aspartate*-specific *protease* (caspase) family members [5]. This coordination is dependent, in large part, on the specificity of the caspase family

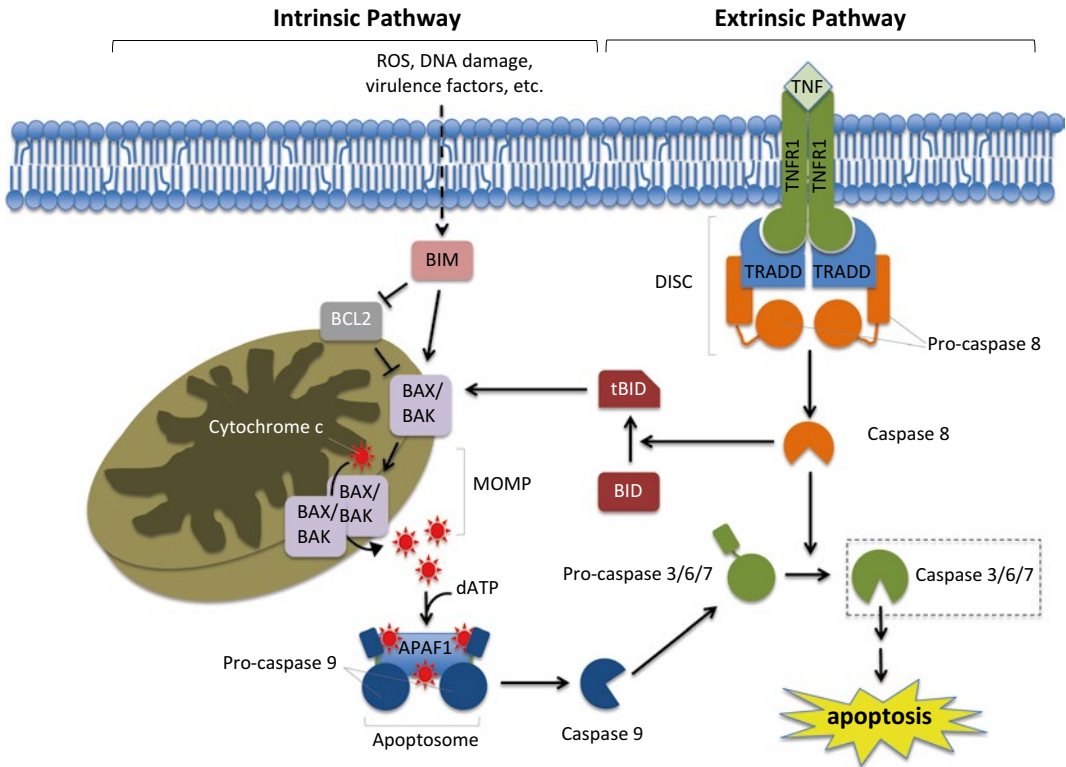


Fig. 1 The intrinsic and extrinsic pathways of apoptosis. Apoptosis can be initiated by either the intrinsic pathway (*left*) or the extrinsic pathway (*right*). The intrinsic, or mitochondrial, pathway is activated in response to a wide variety of cellular stresses, including DNA damage, viral virulence factors, and elevated levels of reactive oxygen species (ROS). This pathway is regulated by both pro- and anti-apoptotic members of the B-cell lymphoma-2 (BCL2) family, including the anti-apoptotic factor BCL2 and the pro-apoptotic factors Bcl2-interacting mediator of cell death (BIM), BCL2 antagonist/killer (BAK) and BCL2-associated X protein (BAX). Dimerization of BAK/BAX leads to mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome c (*red starbursts*) from the mitochondrial intermembrane space into the cytosol. In the cytosol, cytochrome c associates with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 in a dATP-dependent manner to form a large, multimeric complex termed the apoptosome. At the apoptosome, pro-caspase-9 is converted to the active form, leading to cleavage and activation of executioner caspases, such as caspase-3, -6 and -7. Once activated, the executioner caspases process various downstream substrates, ultimately leading to apoptotic cell death. The extrinsic pathway is initiated by cytokines, such as tumor necrosis factor (TNF), which bind the TNF receptor-1 (TNFR1). Ligand binding promotes the activation of TNFR1, leading to its association with TNFR1-associated death domain protein (TRADD). TRADD helps sequester pro-caspase-8 in the death-inducing signaling complex (DISC), which promotes the cleavage and subsequent activation of caspase-8. Caspase-8 cleaves executioner caspases, leading to their activation and the induction of apoptosis. In some cellular contexts, caspase-8 can also cleave the BCL2-homology domain 3 (BH3)-interacting domain death agonist (BID). The truncated form of BID (tBID) then translocates from the cytosol to the mitochondria, where it helps initiate the intrinsic pathway via MOMP. In this way, the extrinsic and intrinsic pathways are linked. It is important to note that both the intrinsic and extrinsic pathways lead to the activation of executioner caspases (*dashed box*). The genetically encoded biosensor, SCAT-3, used in this protocol measures the activity of caspase-3

members for their substrates. Indeed, each caspase family member recognizes and cleaves a specific cleavage motif. For instance, the executioner caspase, caspase-3, recognizes the tetrapeptide sequence Asp-Glu-Val-Asp (DEVD/X) in its substrates, cleaving immediately after the second aspartate residue (represented by a “/”).

In this chapter, we begin with a brief overview of the extrinsic and intrinsic apoptotic pathways before describing a relatively low-cost method to monitor the induction of apoptosis using fluorescence resonance energy transfer (FRET)-based biosensors in a microplate reader format. This method, which can offer insights about the timing of apoptotic induction in different cellular contexts, can also be used to screen a wide variety of pharmacological and toxicological agents for their effect on apoptosis.

1.1 The Intrinsic Pathway

The mitochondrial-dependent intrinsic pathway is activated in response to a wide variety of cellular stresses, including DNA damage, viral virulence factors, and elevated levels of reactive oxygen species (ROS) (Fig. 1a) [3, 6]. This pathway, which is regulated by both pro- and anti-apoptotic members of the Bcl-2 family, is initiated by mitochondrial outer membrane permeabilization (MOMP) [3, 7–9]. MOMP results in the diffusion of various proteins that typically reside in the mitochondrial intermembrane space into the cytosol. Chief among these is cytochrome c, which associates with apoptosis activating factor-1 (Apaf-1) and pro-caspase-9 in a dATP-dependent manner to form a large, multimeric complex termed the apoptosome [10–12]. At the apoptosome, the high local concentration of pro-caspase-9 leads to its autocatalytic conversion to the active form. Once activated, caspase-9 recognizes and cleaves executioner caspases, such as caspase-3, -6 and -7, which process various downstream substrates, leading to apoptotic cell death [5, 12].

1.2 The Extrinsic Pathway

Unlike the intrinsic pathway, which is triggered in response to changes in the intracellular environment, the extrinsic pathway relies upon receptor-mediated signaling to initiate apoptosis (Fig. 1b). In this context, cytokines, such as tumor necrosis factor (TNF), TNF-related apoptosis inducing ligand (TRAIL) and Fas ligand, bind so-called “death receptors” of the TNF receptor (TNFR) superfamily [13–15]. Ligand binding promotes the activation of the death receptors, leading to their association with death domain (DD)-containing adaptor proteins, such as Fas-associated death domain protein (FADD) and TNFR1-associated death domain protein (TRADD) [12, 15]. FADD and TRADD help sequester the zymogenic forms of the initiator caspases, pro-caspase-8 and pro-caspase-10, in the death-inducing signaling complex (DISC) via their death effector domains (DED) [12]. DISC formation leads to the cleavage and subsequent activation of the initiator caspases, which in turn cleave and activate executioner caspases, leading to the induction of apoptosis.

Recent evidence suggests that, in certain cell types (classified as type II cells), insufficient levels of caspase-8 are activated by the DISC to elicit apoptosis. If this is the case, cross talk between the extrinsic and intrinsic pathways via caspase-8-mediated processing of the Bcl-2 family member, BID, leads to apoptosis in a mitochondria-dependent manner (Fig. 1). Under these circumstances, cytosolic BID is cleaved by caspase-8 to generate a 15 kDa BID fragment (tBID) that is redistributed to the mitochondria via the N-myristoylation of an exposed glycine residue at the site of cleavage [16–18]. At the mitochondria, tBID initiates MOMP and the release of apoptogenic factors, such as cytochrome c, that promote the activation of the executioner caspase, caspase-3, and a full apoptotic response via the intrinsic pathway.

In contrast to type II cells, activation of the intrinsic pathway is not formally required for the induction of apoptosis in type I cells, which have been shown to undergo apoptosis despite the overexpression of anti-apoptotic regulators of the intrinsic pathway, such as BCL-2 and BCL-XL [3, 15]. Nonetheless, it is important to note that, even in type I cells, cross talk between the extrinsic and intrinsic pathways routinely occurs, serving to amplify and reinforce apoptotic initiation. Thus, in both type I and type II cells, caspase-3 activation is associated with induction of the apoptotic response. As a consequence, caspase-3 activity is an attractive marker of apoptosis induction [19]. Here, we describe a low-cost method to track changes in caspase-3 activity in real-time within the native cellular environment using the genetically encodable FRET-based biosensor, sensor of activated caspase-3 using FRET (SCAT-3) [20].

1.3 Genetically Encoded Biosensors for Measuring Induction of Apoptosis in Living Cells

Genetically encodable FRET-based reporters, which are able to track the dynamics of a variety of signaling molecules in the native cellular environment with high spatial and temporal resolution, have traditionally been used in conjunction with live cell imaging to better understand the regulation of complex cellular pathways [21, 22]. To date, FRET-based biosensors have been used to study the dynamic regulation of a wide range of cellular signaling molecules, including key second messengers (such as Ca^{2+} [23–26] and cAMP [27–31]), important signaling enzymes (such as protein kinases [32], phosphatases [33, 34] and small G-proteins [35–39]), and cell surface receptors (such as G-protein-coupled receptors [40] and receptor tyrosine kinases [41–43]) (reviewed in Refs. [21, 44]). However, due to the high cost of the fluorescence microscopy systems typically used for live cell imaging, these molecular tools are not readily accessible to many researchers. Recently, methods have been developed to monitor various cellular parameters utilizing FRET-based reporters using a microplate reader format [45–47]. For instance, Robinson et al. recently described a method to conduct live cell compound screens using

FRET-based biosensors of the cAMP-PKA signaling pathway [45]. Here, we describe a low-cost strategy to monitor the induction of apoptosis in non-adherent TK6 lymphoblasts using the caspase-3 activity reporter, SCAT-3, in conjunction with a fluorescent microplate reader.

1.4 Biosensor and Drugs Used in This Protocol

SCAT-3, which was constructed by sandwiching a short peptide containing the caspase-3-specific recognition sequence, DEVD, between enhanced cyan fluorescent protein (ECFP) and the yellow fluorescent protein, Venus, has been used to monitor the induction of apoptosis in many cellular contexts using live cell fluorescence microscopy (Fig. 2a) [19, 20, 48]. Due to the high degree of spectral overlap between the emission profile of ECFP (i.e., the donor) and the excitation profile of Venus (i.e., the acceptor), these fluorescent proteins (FPs) are able to undergo efficient FRET when they are in close proximity to one another (Fig. 2b, blue line). However, following caspase-3-mediated cleavage of the biosensor, the ECFP-Venus FP FRET pair is no longer linked together, resulting in a dramatic reduction in FRET as they diffuse away from one another (Fig. 2b, red line). In this way, SCAT-3 is able to track the activation of caspase-3 in real-time under a variety of cellular conditions. It is important to note that enzymatic amplification of the signal, coupled with the large change in FRET caused by biosensor cleavage, allows SCAT-3 to detect increases in caspase-3 activity associated with apoptosis at much earlier time points than traditional end-point assays, such as DNA fragmentation assays. On the other hand, unlike FRET-based biosensors of other cellular processes that typically rely on a signal-dependent conformational change in the biosensor to alter FRET, the irreversible nature of cleavage means that SCAT-3 and related FRET-based protease activity sensors are unable to report on the attenuation of the signal. Nonetheless, such FRET-based protease biosensors have proven to be powerful tools for monitoring changes in protease activity under a variety of cellular conditions, particularly during apoptosis.

Here, we use diepoxybutane (DEB)-induced apoptosis in SCAT-3 transfected TK6 human lymphoblasts as an example of how FRET-based biosensors can be used in a microplate reader format to monitor the induction of apoptosis (Fig. 3). DEB, which is the most toxic metabolite of the high production volume industrial chemical 1,3-butadiene, has been shown to induce cell death in TK6 lymphoblasts via the production of ROS and activation of the intrinsic apoptotic pathway [49].

We begin by discussing strategies for transfection of the biosensor DNA. We then outline the steps for performing the FRET assay and end with tips for data analysis. Though the details provided are specific for non-adherent TK6 human lymphoblasts, the protocol can be applied to numerous cell lines. Moreover, though

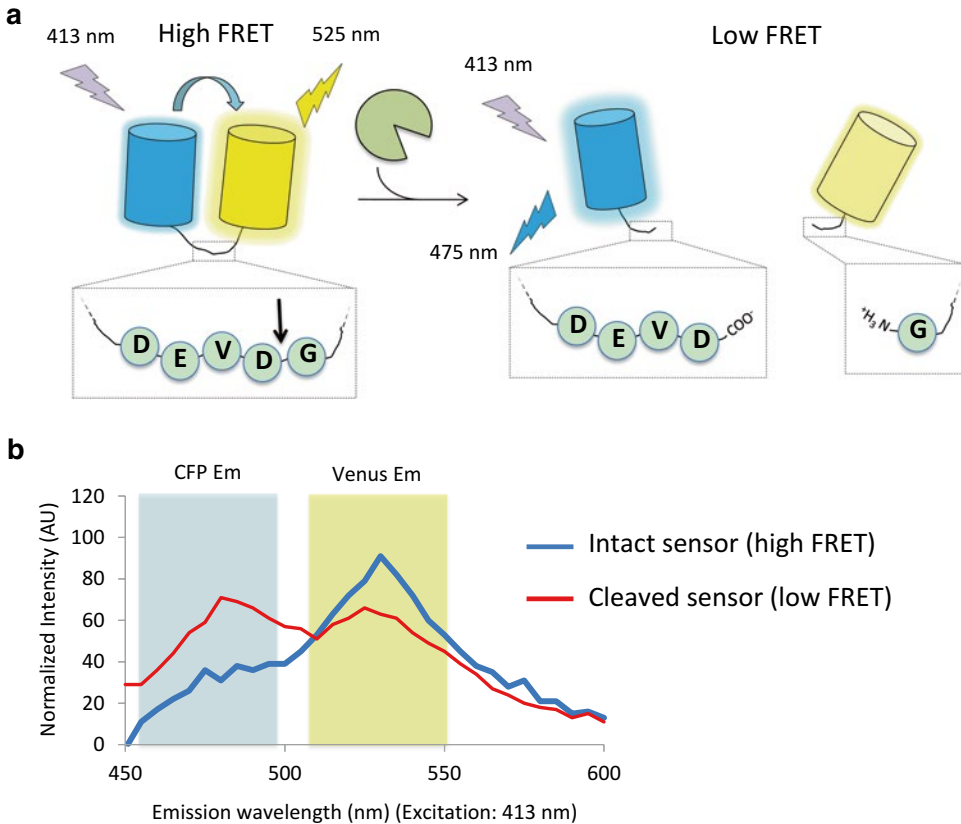


Fig. 2 Overview of the caspase-3 activity sensor, SCAT3. **(a)** The genetically encoded FRET-based biosensor, SCAT3, is composed of the caspase-3 recognition sequence (DEVD) sandwiched between the FP FRET pair, enhanced CFP (ECFP; *cyan cylinder*) and Venus (*yellow cylinder*). When the sensor is intact (*left side*), ECFP and Venus are in close proximity to one another. As a consequence, excitation of ECFP with 413 nm light (*light purple lightning bolt*) leads to non-radiative transfer of the excited state energy to Venus via FRET (*curved arrow*), causing emission at 525 nm (*yellow lightning bolt*). Caspase-3-mediated cleavage of the sensor causes an increase in the distance between the FP's, leading to reduced FRET between ECFP and Venus. As a consequence, excitation at 413 nm leads to emission by ECFP at 475 nm (*cyan lightning bolt*). **(b)** Representative SCAT3 emission scans from 450 to 600 nm following excitation at 413 nm before (*blue line*) and after (*red line*) caspase-3 activation. The regions of the spectrum used to quantitate ECFP emission (*cyan box*; 455–495 nm) and Venus emission (*yellow box*; 515–545 nm) are shown

we discuss only DEB as an apoptosis-inducing agent, the protocol can be used to measure the dynamics of apoptosis induction for a variety of xenobiotics (provided that their fluorescence spectra do not interfere with FRET measurements). Likewise, the protocol also can be easily adapted to screen compound libraries in order to assess the impact of a large number of compounds on the induction of apoptosis.

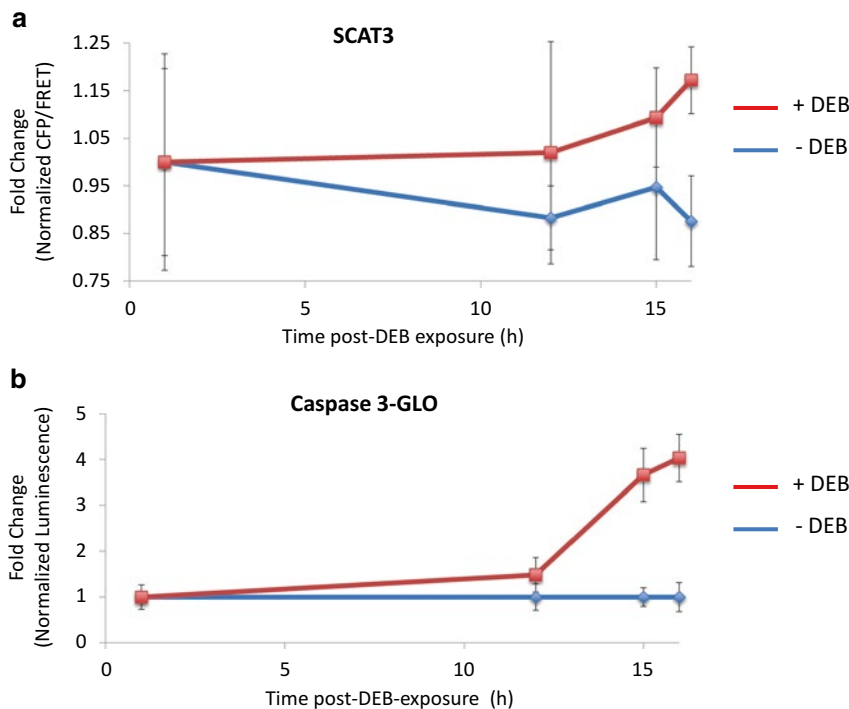


Fig. 3 Time course of DEB-induced apoptosis measured by orthogonal SCAT-3 and Caspase-3 GLO assays. Time course of DEB-induced apoptosis in TK6 lymphoblasts measured using SCAT3 (**a**) and Caspase-3 GLO (**b**). In both (**a**) and (**b**), the signal from cells treated with DEB are shown in *red* while that from untreated samples is shown in *blue*

2 Materials

2.1 Cell Culture

1. The TK6 human lymphoblastic cell line (The American Type Culture Collection Catalogue # CRL 8015).
2. Roswell Park Memorial Institute 1640 culture media (RPMI 1640, Gibco/BRL, Bethesda, MD) supplemented with 10 % fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA).
3. T150 tissue culture flask.
4. 6-well tissue culture dishes.
5. 96-well assay plate, black walled, clear bottom with lid, polystyrene, sterile (Costar, No. 3603).

2.2 Transfection Materials

1. Biosensor DNA (e.g., SCAT3 and SCAT3-DEVG), kindly provided by Prof. Masayuki Miura (**Note 1**).
2. RPMI-1640 cell culture medium (Gibco/BRL, Bethesda, MD) supplemented with 10 % FBS.
3. Amaxa Nucleofector device with 96-well shuttle.
4. Nucleofector kit containing Nucleofection solution SF (Lonza, Walkersville, MD).

Table 1
The Infinite M200 PRO instrument settings for an emission scan following 413 nm excitation

Mode	Fluo top scan
Emission Wavelength Start	450 nm
Emission Wavelength End	600 nm
Emission Wavelength Step	2 nm
Emission Scan Number	76
Excitation Wavelength	413 nm
Bandwidth (Em)	280...850: 20 nm
Bandwidth (Ex) (Range 1)	230...315: 5 nm
Bandwidth (Ex) (Range 2)	316...850: 10 nm
Gain	Optimal
Number of Flashes	10
Integration Time	20 μ s
Lag	0 μ s
Settle Time	0 ms
Z-Position (Manual)	20,000 μ m

2.3 Cell Counting

1. Hemocytometer.
2. 0.4 % trypan blue (Life Technologies, Carlsbad, CA).

2.4 FRET Assay Materials and Devices

1. Hank's Balanced Salt Solution (HBSS): 0.15 M NaCl, 4 mM KCl, 1.2 mM MgCl₂·6H₂O, 55.5 mM glucose, 20 mM HEPES–KOH, pH 7.2; Store at 4 °C.
2. Tecan Infinite M200 PRO fluorescence microplate reader (Tecan, Inc.) set to conduct an emission scan following excitation at 413 nm (**Note 2**). The settings for the emission scan are given in Table 1.
3. Diepoxybutane (DEB; Sigma): 10 mM DEB stock solution (1000 \times). CAUTION: DEB is a potent carcinogen. Care must be taken when handling this reagent.

2.5 Data Analysis

1. Spreadsheet application (e.g., Microsoft Excel).

3 Methods

3.1 Cell Culture and Transfection

1. Propagation of cell cultures. TK6 cells are routinely propagated at a density of 2×10^5 cells/ml in RPMI-1640 medium containing 2 mM glutamine and 10 % FBS. Cells are passaged every 36 h when the density reaches 2×10^6 cells/ml, and are used for experiments at 12 h after they are passaged onto fresh medium.
2. Determine the distribution of experimental and control samples. Table 2 highlights suggested experimental and control samples, the biosensor to be transfected (if any) under each condition, the treatment condition, and the reason that each condition is included.
3. In a T150 tissue culture flask, grow 120 ml of TK6 human lymphoblasts to a density of $\sim 1 \times 10^6$ cells/ml at 12 h prior to transfection (**Note 3**).
4. Transfect the cells via nucleofection (**Note 4**).
 - (a) Determine the total number of cells to be nucleofected, as well as the number of nucleofection reactions to be conducted (**Note 5**).
 - (b) Pellet the cells at $200 \times g$ for 10 min (**Note 6**).
 - (c) While the cells are being pelleted:
 - Start the Nucleofector 96-well shuttle software and generate a parameter file from the predefined template for cell line optimization (for TK6 lymphoblasts, we use Program DS 137).
 - In the CO₂ incubator, pre-warm 6-well plates containing 2 ml of culture media per well.

Table 2
Sample experimental layout

Condition	Biosensor	Treatment	Parameter
1	None	None	Reference set for untreated cells
2	None	DEB	Reference set for treated cells
3	SCAT3	None	Untreated, SCAT3 control set
4	SCAT3	DEB	Experimental set
5	SCAT3-DEVG	None	Untreated, SCAT3-DEVG control set
6	SCAT3-DEVG	DEB	DEB-treated, SCAT3-DEVG control set

SCAT3: FRET-based caspase-3 sensor; SCAT3-DEVG: control construct in which the caspase-3 cleavage site, DEVD, has been mutated to DEVG; DEB: diepoxybutane

- In a 37 °C water bath, pre-warm a separate aliquot of culture medium (use 200 µl of media per nucleofection reaction).
 - Pre-warm Nucleofector solution SF to room temperature.
 - For each nucleofection reaction, add 400 ng of the appropriate biosensor DNA to a sterile PCR tube in a strip format (this will allow a multichannel pipette to be used in subsequent transfection steps).
- (d) Following centrifugation, carefully decant the supernatant. Remove supernatant completely (**Note 7**).
 - (e) Gently resuspend the cell pellet in Nucleofector solution SF to a final concentration of 1×10^6 cells per 21 µl (e.g., for 120×10^6 cells, resuspend cells in 2.52 ml of Nucleofector solution SF). Add the cell suspension to a sterile small volume multichannel pipette reservoir. Avoid creating bubbles at all times when working with cells in nucleofection solution.
 - (f) Using a multichannel pipette, add 21 µl of the resuspended cells (i.e., 1×10^6 cells) to each PCR tube containing DNA and mix by pipetting up and down several times; avoid creating bubbles. Using the same pipet and tips, transfer 20.5 µl of the DNA/cell suspension to the appropriate nucleofection cuvette wells, taking care to deliver the DNA/cell suspension to the bottom of the cuvettes.
 - (g) Put the lid onto the 96-well shuttle plate, and gently tap the cuvette to ensure that the sample covers the bottom of the cuvettes.
 - (h) Put the 96-well shuttle plate into the nucleofector device and start the nucleofection process by pressing “upload and start” in the shuttle software.
 - (i) Following nucleofection, open the retainer and carefully remove the 96-well cuvette strips from the retainer.
 - (j) Add 100 µl of pre-warmed culture media to each cuvette and transfer the cells into the corresponding well of the 6-well plates. To increase recovery of cells, repeat once more.
 - (k) Incubate the nucleofected cells at 37 °C for 12 h in a CO₂ incubator.
5. Measure the transfection efficiency 12 h after transfection. Proceed only if greater than 50 % transfection efficiency is achieved (**Note 8**).

3.2 Induction of Apoptosis and Fluorescence Data Acquisition

On the day of the experiment, pool all cells containing the same biosensor (e.g., all cells expressing SCAT3) and measure the density of the pooled cells using a hemocytometer or automated cell counting device. If using a hemocytometer:

- Add 10 μl of cells to 10 μl of 0.4 % trypan blue.
- Add the solution to the hemocytometer and count all of the unstained cells in one quadrant (large square).
- Determine the number of live cells per ml according to the following equation (**Notes 9** and **10**):

$$\text{Cells / ml} = (\# \text{ cells}) \times (\text{dilution factor (e.g., 2)}) \times 10^4 / \text{ml}$$

1. Prior to DEB addition, dilute each set of pooled cells to a density of 2.0×10^5 cells/ml using pre-warmed RPMI, 10 % FBS culture media.
2. Split each set into two groups. The first group, which will be an untreated control, will contain 40 % of the pooled cells. The second group, which will be treated with DEB, will contain 60 % of the pooled cells. This is done in order to account for the fact that DEB inhibits cell proliferation.
3. Add DEB to the cells according to the experimental scheme outlined in Table 1. Add DEB to a final concentration of 10 μM with gentle agitation to ensure even distribution of the xenobiotic (**Note 11**). Unexposed control cells should receive vehicle only (culture medium, in this case).
4. Measure the baseline fluorescence immediately after DEB addition.
 - (a) Remove 2.4×10^6 cells from each group and pellet the cells at $800 \times g$ for 3 min. While the cells are being pelleted, place the remaining cells back in the CO_2 incubator.
 - (b) Following centrifugation, remove the media taking care not to disturb the cell pellet (**Note 11**).
 - (c) Wash each pellet once with 1/5 volume of HBSS imaging buffer (e.g., if 6 ml of cells in culture were initially pelleted, the cell pellet should be washed with 1.2 ml of HBSS).
 - (d) Pellet the cells again at $800 \times g$ for 3 min.
 - (e) Carefully remove the supernatant and gently resuspend each cell pellet in 330 μl of HBSS imaging buffer.
 - (f) Transfer $3 \times 100 \mu\text{l}$ of the cell suspension to the appropriate well in the 96-well black-walled assay plate.

- (g) Insert the 96-well plate into the microplate reader and conduct an emission scan in each well using 413 nm excitation (please refer to Table 1 in Sect. 2.4, item 2 for microplate reader settings) (**Note 12**).
5. At each time point, remove 2.4×10^6 cells and conduct an emission scan using an excitation wavelength of 413 nm, as outlined in step 4 (**Note 13**).

3.3 Data Analysis

1. The output of an emission scan will be a series of data points corresponding to the fluorescence emission at each wavelength across the designated scan range. For example, using the settings shown in Table 1, the fluorescence emission following excitation at 413 nm will be measured at 2 nm intervals from 450 to 600 nm (for a total of 76 data points per well).
2. Using a spreadsheet application (e.g., Microsoft Excel), calculate the average fluorescence at each wavelength in the wells that contained non-transfected cells. For example, calculate the average fluorescence at each wavelength for the non-transfected, untreated cells. Do the same for the non-transfected, DEB-treated cells. These values represent the background fluorescence with and without DEB treatment.
3. Subtract the background fluorescence at each wavelength from the analogous value for each condition. Be sure to use the appropriate reference set for background correction (e.g., pair “untreated with untreated” and “treated with treated”). These values serve as the background corrected values.
4. To determine the emission intensity directly from CFP (i.e., CFP direct), add all of the background corrected values from 455 to 495 nm (this corresponds to a 475 nm emission peak with a 20 nm bandwidth). To determine the emission intensity from YFP FRET (i.e., YFP FRET), add all of the background corrected values from 505 to 545 nm (this corresponds to a 525 nm emission peak with a 20 nm bandwidth).
5. For each time point, take the ratio of the CFP direct (i.e., donor) to YFP FRET (i.e., acceptor) according to the following equation:

$$\text{Emission Ratio} = \frac{\text{Background corrected CFP direct}}{\text{Background corrected YFP FRET}}$$

6. Normalize the calculated ratio at each time point to the average ratio of the corresponding set immediately following DEB addition (i.e., t_{1h}). This represents the fold change at each point.
7. Calculate the average fold change and standard error at each point.
8. To obtain a time course of DEB-induced apoptosis, plot the normalized ratios over time (Fig. 3).

4 Notes

1. Early versions of FRET-based caspase sensors employed enhanced yellow fluorescent protein (EYFP) as the FRET acceptor rather than the next generation YFP, Venus [50–52]. Because EYFP is acutely sensitive to changes in pH and the Cl^- concentration, changes in the cellular environment not directly related to caspase-3 activity could quench EYFP fluorescence, leading to an apparent reduction in FRET in the absence of sensor cleavage. Though Venus fluorescence is not affected by either pH or Cl^- at physiologically relevant levels, SCAT3-DEVG may be employed as a negative control in these experiments. SCAT3-DEVG contains a modified tetrapeptide sequence in which the caspase-3-specific cleavage site, DEVD, has been mutated to DEVG. As a consequence, SCAT3-DEVG is not cleaved by caspase-3. Likewise, SCAT9, which contains the caspase-9-specific cleavage motif, LEHD, in place of DEVD, is also available. SCAT9 specifically monitors caspase-9 activity.
2. The Infinite M200 PRO is a monochromator-based “filterless” microplate reader. If a filter-based system is used, then the following filters should be used: one 420DF20 excitation filter and two emission filters (470DF40 for donor CFP and 535DF25 for acceptor Venus).
3. Here, we describe a protocol using TK6 lymphoblasts as an example. Optimization of cell culture and transfection techniques, as well as plating density if adherent cells are used, may be necessary for other cell types. For TK6 lymphoblasts, this cell density should be sufficient for five time points at 8.0×10^5 cells per well measured in triplicate. If additional time points are desired, the number of cells should be scaled accordingly. The total DNA should also be scaled accordingly.
4. All transfection steps should be carried out in a sterile biosafety cabinet.
5. For each condition (e.g., SCAT3 + DEB), approximately 12.0×10^6 cells will be required. To maintain consistency, treated and non-treated cells containing the same biosensor should be derived from the same population of transfected cells. Therefore, at the time of transfection, approximately 24×10^6 cells should be transfected with each biosensor (12×10^6 cells per condition \times 2 conditions). Since 1×10^6 cells are used per nucleofection reaction, this would require a total of 24 nucleofection reactions.
6. TK6 lymphoblasts are non-adherent suspension cells. If using adherent cells for nucleofection, the cells must first be trypsinized prior to centrifugation. Other transfection procedures that are effective for the cell type under study can also be utilized.

7. Be sure not to aspirate off the pellet. It is best to turn the centrifuge tube horizontally to aspirate the media off of the side of the tube, keeping the tip away from the pellet.
8. If the transfection efficiency is <50 %, then the signal-to-noise ratio may be too low to achieve high sensitivity. If transfection efficiencies >50 % cannot be achieved, a cell line stably expressing the biosensor-of-interest can be generated and used as an alternative. Likewise, if using a fluorescent biosensor with a signal-to-noise ratio lower than that of SCAT3, higher transfection efficiencies may be necessary.
9. Live TK6 lymphoblasts will appear round and translucent while dead cells will appear blue and may be irregularly shaped. Count only the live cells.
10. The volume of one square on the hemocytometer is 0.1 mm³ (or 1.0×10⁻⁴ ml) so the quick calculation is: cells/ml = # cells × Dilution Factor × 10⁴/ml. For more accurate cell counts, count the number of cells in two to four quadrants (large squares) and use the average to calculate the cell density
11. DEB is a potent carcinogen. Care should be taken when working with DEB-treated cells and any by-products (e.g., supernatants) from the experiments should be disposed of in accordance with institutional, state and federal regulations.
12. If a need to verify assay performance arises, an orthogonal detection assay, such as the bioluminescence-based Caspase 3-GLO assay (Promega, Madison, WI), may be conducted according to the manufacturer's instructions once the emission scan has been completed.
13. If the microplate reader being used for the experiments is equipped with temperature and CO₂ control modules, continuous measurements can be obtained (e.g., at 1 h intervals for 36 h) by substituting RPMI-1640 media lacking phenol red for the standard RPMI-1640 media.

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