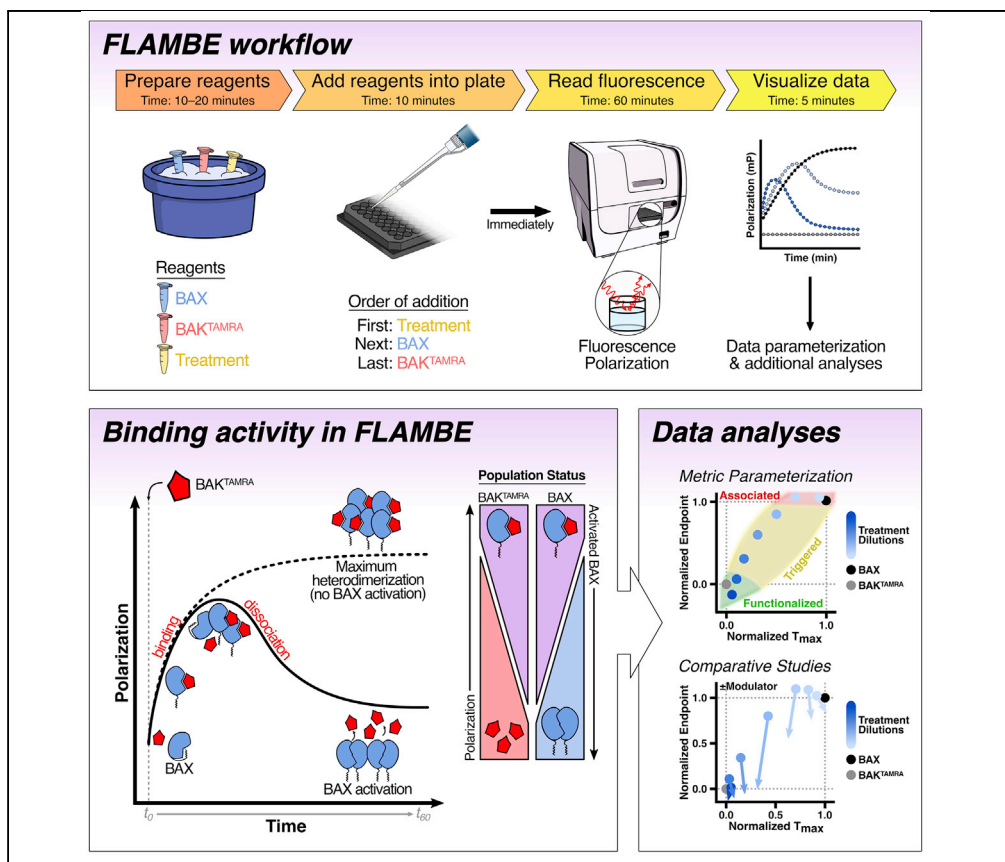


Protocol

FLAMBE: A kinetic fluorescence polarization assay to study activation of monomeric BAX



BAX activation techniques are crucial to studying the intrinsic pathway of apoptosis – thousands of pro-apoptotic signals converge on BAX activation. Current methodologies are predominantly limited to membrane permeabilization studies, which assess endpoint functionality of oligomeric BAX, but overlook early activation steps of cytosolic BAX. Here we detail FLAMBE: a fluorescence polarization ligand assay for monitoring BAX early-activation in solution. We also describe a dual-metric parameterization strategy for distillation of kinetic data and comparative analyses when studying candidate ligands.

Jarvier N. Mohammed, Jesse D. Gelles, Jerry Edward Chipuk

jarvier.mohammed@icahn.mssm.edu (J.N.M.)
jerry.chipuk@mssm.edu (J.E.C.)

Highlights

FLAMBE is a fluorescence polarization assay to detect activation of recombinant BAX

FLAMBE detects monomeric BAX activation via release of a labeled BAK-BH3 peptide

Parameterized data identifies conditions achieving distinct functional states of BAX

FLAMBE comparative analyses serve as a platform to study modulation of BAX activation

Mohammed et al., STAR Protocols 3, 101252
June 17, 2022 © 2022 The Author(s).
<https://doi.org/10.1016/j.xpro.2022.101252>



Protocol

FLAMBE: A kinetic fluorescence polarization assay to study activation of monomeric BAX

Jarvier N. Mohammed,^{1,2,3,4,6,7,8,*} Jesse D. Gelles,^{1,2,3,4,7} and Jerry Edward Chipuk^{1,2,3,4,5,6,9,*}

¹Laboratory of Mitochondrial Biology in Human Health and Disease, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

²Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

³Department of Dermatology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

⁴The Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

⁵The Diabetes, Obesity, and Metabolism Institute, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

⁶The Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

⁷These authors contributed equally

⁸Technical contact

⁹Lead contact

*Correspondence: jarvier.mohammed@icahn.mssm.edu (J.N.M.), jerry.chipuk@mssm.edu (J.E.C.)
<https://doi.org/10.1016/j.xpro.2022.101252>

SUMMARY

BAX activation techniques are crucial to studying the intrinsic pathway of apoptosis— thousands of pro-apoptotic signals converge on BAX activation. Current methodologies are predominantly limited to membrane permeabilization studies, which assess endpoint functionality of oligomeric BAX, but overlook early activation steps of cytosolic BAX. Here we detail FLAMBE: a fluorescence polarization ligand assay for monitoring BAX early-activation in solution. We also describe a dual-metric parameterization strategy for distillation of kinetic data and comparative analyses when studying candidate ligands. For complete details on the use and execution of this protocol, please refer to Gelles et al. (2022).

BEFORE YOU BEGIN

This section describes the steps for preparing and storing aliquots of assay reagents as well as setting up a detection procedure using a BioTek Synergy H1 Hybrid Multi-Mode Plate Reader.

Prepare assay reagents

⌚ Timing: 30 min

⚠ **CRITICAL:** Multiple detergents (e.g., CHAPS, Triton X-100, SDS) have been demonstrated to affect BAX conformation and activity, even at nanomolar concentrations (Hsu and Youle, 1997). Detergent contamination commonly arises when preparing buffers using shared glassware that is often cleaned with detergents. Therefore, we suggest using separate materials and labware when preparing reagents necessary for this assay.

1. Prepare single-use aliquots (10–25 μ L) of recombinant human BAX protein at a concentration of 10–30 μ M (storing BAX at higher concentrations may risk aggregation and precipitation over time)



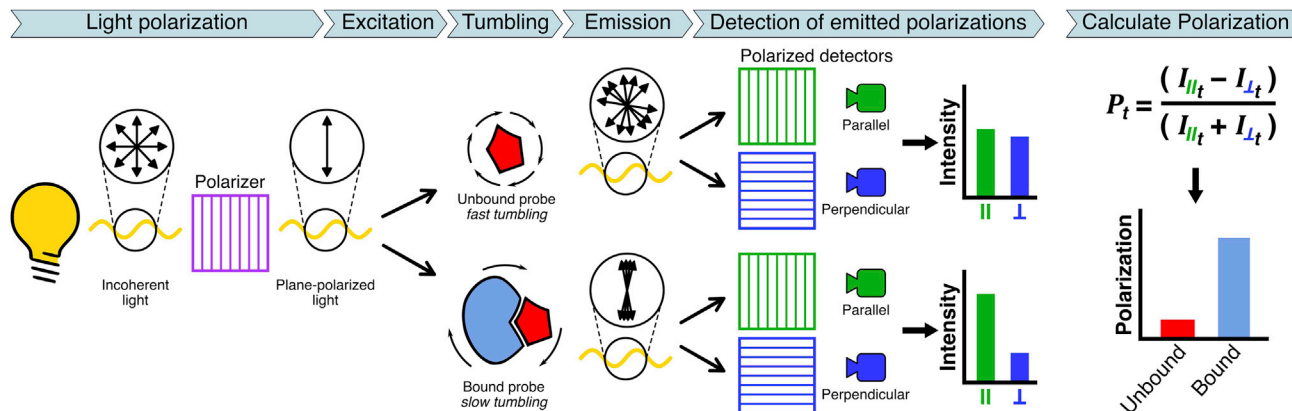


Figure 1. Principle of a kinetic FP assay

Plane-polarized light is used to excite a fluorescent ligand. In the unbound state, the ligand tumbles faster in solution and emits light in several polarizations that is recorded by parallel and perpendicular polarizers. In the bound state, the increase in molecular volume of the ligand results in slower tumbling and light is emitted in fewer polarizations that are more parallel to the exciting plane-polarized light. The metric, Polarization (P), is calculated from the parallel (\parallel) and perpendicular (\perp) intensities (I) for each time point (t). Data are reproduced and adapted from Gelles et al. (2022).

resulting in confounding observations; see [problems 3](#) and [4](#) in the [troubleshooting](#) section). Flash freeze in liquid nitrogen and store aliquots at -80°C .

△ CRITICAL: Use necessary PPE when handling liquid nitrogen.

2. Reconstitute TAMRA-labeled BAK-BH3 peptide ($\text{BAK}^{\text{TAMRA}}$) at a stock concentration of 0.5 mM in DMSO. Prepare 2.2 μL single-use aliquots in amber tubes and protect from light. Store at -80°C .
3. Using detergent-free materials, prepare 200 mL of 0.5 \times PBS in ultrapure water. Store at 4°C .

Note: We have determined that the salt concentration of 0.5 \times PBS is optimal for facilitating binding and release of $\text{BAK}^{\text{TAMRA}}$ from BAX. 200 mL of 0.5 \times PBS is in large excess and may be used for an extended period if kept at 4°C . For consistency with downstream assays, alternative BAX-compatible assay buffers may be used but this may affect data trends (see [limitations](#) section).

4. Reconstitute BIM-BH3 peptide (BIM^{BH3}) at a stock concentration of 1 mM in DMSO. Prepare 5 μL aliquots and store at -80°C .

Set up a fluorescence polarization (FP) measurement protocol

⌚ Timing: 10–20 min

FP assays are a type of fluorospectrometric technique that detect molecular interactions by measuring changes in polarized light. An FP assay uses a polarizer to generate “plane-polarized light” (consisting of a single polarization) from a traditional illumination source, which produces light consisting of several polarizations. The plane-polarized light excites a fluorescent probe, which then emits light consisting of several polarizations due to random rotation (“tumbling”) during the fluorescence lifetime (Figure 1). The degree of depolarization from the initial plane-polarized light is proportional to the rate of tumbling and therefore the molecular volume of the probe. When the probe is bound, the increased molecular volume results in slower tumbling and light is emitted with less polarization diversity. Emission intensity (I) is measured through parallel and perpendicular polarizers and may be expressed as a fractional ratio called Polarization (P), which represents the ratio of divergence from the initial plane-polarized light. The Polarization value of a whole sample is

the weighted average of individual emissions from bound and unbound probes and may be used to approximate the population fraction of bound probe. Of note, the Polarization of the probe is inversely proportional to the concentration and therefore investigators should select a concentration that provides adequate signal resolution compared to buffer-alone control, which marks the limiting Polarization value for a particular assay setup.

Below are the steps for creating an FP protocol using the Gen5 software with a BioTek Synergy H1 Hybrid Multi-Mode Plate Reader. Investigators using plate readers from other companies should consult the manufacturer's instructions for creating analogous protocols to measure fluorescence polarization.

5. Launch the Gen5 software on the computer connected to the Synergy H1 reader. This will automatically open the software's Task Manager window. From the left menu of the Task Manager window, select "Protocols". On the right menu, select "Create new".
6. In the pop-up menu, select "Standard protocol". This will create a "File" menu on the left for options to edit the protocol's Procedure, Plate Layout, Data Reduction, and Report/Export Builders.
7. Install the fluorescence polarization cube according to the manufacturer's instructions (if needed). In this execution of the protocol, we use a red FP filter cube (BioTek Cat#: 8040562) with the following parameters: Ex: 530/25, Em: 590/35, Dichroic: 570 nm.
8. *Procedure*. Define the parameters of the measurements.
 - a. From the File menu on the left, select Procedure. Set Plate Type to "Costar 96 black opaque", or the appropriate setting for an analogous plate. Check the option for selecting wells at runtime and ensure "Use lid" is unchecked.

Note: This assay is performed at ambient temperature (~25°C). While an assay temperature is not required to be specified for the protocol, investigators may elect to set the temperature to 25°C. Importantly, this "Set Temperature" step must be placed before the "Start Kinetic" step.

- b. From the left "Select steps" menu, select "Start kinetic" under the Kinetic header.
 - i. Set "Run" to 1 h. Set "Interval" to 30 s. The software will automatically calculate 121 reads. Click "OK".

Note: Due to the importance of the binding kinetics of this assay, we suggest a 30-second interval to allow for denser data collection. This permits more reads and generation of kinetic data with finer resolution.

- c. From the "Select steps" menu, select "Shake" under the Actions header.
 - i. Set "Shake mode" to Double Orbital. Set "Duration" to 3 s and Linear frequency" to 282 cpm (3 mm) on the slider. Click "OK".
 - d. From the "Select steps" menu, select "Read" under the Actions header.
 - i. Select "Fluorescence polarization". Endpoint/Kinetic and Filters should now be auto-selected under Read Type and Optics Type, respectively. Click "OK".
 - ii. The Read Step (Kinetic) menu will now open. From the Filter Set dropdown menu, select your FP cube (defined in step 7). Set Gain to 50.

Note: Alternative machines may have different sensitivities for detecting emission Intensity. The gain should be adjusted so that the parallel and perpendicular Intensity measurements fall within the plate reader's detectable range.

- iii. Ensure Filter Switching per well is unchecked. Set Read Speed to Normal. Set Read Height to 7.00 mm, or appropriate for your plate. Click "OK".

△ **CRITICAL:** In the "Description" section on the right, ensure the Shake and Read steps are indented between the Start and End Kinetic steps.

9. **Data Reduction.** Check the protocol's data reduction steps. These will be auto-generated based on the Procedure steps.
 - a. From the File menu on the left, select Data Reduction.
 - b. Select "Fluorescence Polarization".
 - i. Parameters for Label, Parallel Intensity, and Perpendicular Intensity are default.
 - ii. Under "Select the data to show", ensure boxes for the following are checked: Parallel Intensity, Perpendicular Intensity, and Polarization. Click "OK".

Note: The machine measures parallel and perpendicular Intensities to calculate Polarization, which is the reported metric used for data analysis. Investigators may wish to inspect the raw Intensity data for quality control purposes.

 - c. Remove "Kinetic Analysis" by right clicking and selecting "Remove". Click "OK".
10. **Report/Export Builders.** Define the protocol's data export settings.
 - a. From the File menu on the left, select Report/Export Builders.
 - b. Select "New export to file...".
 - c. From the left menu, select Properties. Under Content, select Custom.
 - d. From the left menu, select Content.
 - i. (Optional) Add Procedure Summary and Data Reduction Summary.
 - ii. Expand the "Table" menu and select Well Data. Add Polarization. This will launch a pop-up window. Ensure Selection by well is checked and in the "Wells" field type "A1-H12". Click "Add". The window on the right will populate with all wells in a 96-well plate. Click "OK".
 - e. From the left menu, select Options. Here investigators may choose options for data file generation and export. For accessibility, we recommend setting the export file type to either a .txt or a .csv file. Click "OK". Click "Close" on the Report/Export Builders window.
11. Save the protocol by clicking the blue floppy disk at the top of the Gen5 window.

Launch the FP assay protocol

⌚ **Timing:** 1 min

To capture initial rapid binding kinetics, it is recommended to launch the protocol before the assay plate is prepared. This will ensure that the assay is ready to run as soon as the BAK^{TAMRA} probe is added to the plate. This is particularly important for capturing and resolving the kinetics of BAK^{TAMRA} binding and dissociation (see [problem 7](#) in the [troubleshooting](#) section).

12. Launch the FP protocol.
 - a. If the protocol is still open, select the green Play button to run the assay.
 - b. If the Gen5 software is closed, launch the software. From the left menu of the Task Manager window, select Read Now and run the FP protocol created in the previous section from the "Existing protocol..." option.
13. Highlight the experimental wells in the "Plate 1 Reading" window that appears. Click "OK".

Note: Consecutive wells must be selected. Additionally, in the interest of collecting denser kinetic data from this assay, it is recommended that experimental conditions be restricted to duplicates and a third column be used only for experimental controls. See [Figure 3](#) and the [limitations](#) section for more details.

14. Designate a name and file location for saving the experiment file. This file will contain the experimental data and is distinct from the data export file that will be generated at the end of the assay.

- Click "OK". The tray should eject automatically, and the "Load Plate" window should appear. Verify that "Use Lid" is unchecked.

▮▮ Pause point: See the [step-by-step method details](#) section for instructions on how to set up an assay. Once the assay is prepared and BAK^{TAMRA} is added, immediately load the plate onto the tray and click "OK" to run the protocol. The assay will run for 1 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Human BAK-BH3, 5-TAMRA-labeled ("BAK ^{TAMRA} ")	AnaSpec	Cat#: AS-64590
Human BAX	N/A	Accession: NP_620116
Human BIM-BH3, Peptide IV ("BIM ^{BH3} ")	AnaSpec	Cat#: AS-62279
Potassium Chloride	Sigma-Aldrich	Cat#: P3911
Potassium Phosphate Monobasic	Sigma-Aldrich	Cat#: P0662
Sodium Chloride	Sigma-Aldrich	Cat#: S9888
Sodium Phosphate Dibasic	Sigma-Aldrich	Cat#: S9763
Recombinant DNA		
pTYB1.BAX	Suzuki et al. (2000)	N/A
Software and algorithms		
Gen5 v3.10	Agilent/BioTek	https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/overview/
Microsoft Excel v16.58	Microsoft	https://www.microsoft.com/en-us/microsoft-365/excel
Prism v9.3.1	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
8-channel multi-channel pipette (20–200 μ L)	Gilson	Cat#: F144072
96-well PCR plate (as reagent reservoir)	Thermo Fisher Scientific	Cat#: AB-1400L
96-well solid black polystyrene microplate	Corning	Cat#: 3915
BioTek Red FP filter set	Agilent/BioTek	Cat#: 8040562
BioTek Synergy H1 Hybrid Multi-Mode Plate Reader	Agilent/BioTek	https://www.biotek.com/products/detection-hybrid-technology-multi-mode-microplate-readers/synergy-h1-hybrid-multi-mode-reader/

MATERIALS AND EQUIPMENT

0.5 \times PBS (pH 7.4)	Final concentration	Amount
Sodium Chloride (mw: 58.4 g/mol)	0.137 M	0.8 g
Potassium Chloride (mw: 74.55 g/mol)	0.0027 M	0.02 g
Sodium Phosphate Dibasic (mw: 141.96 g/mol)	0.01 M	0.144 g
Potassium Phosphate Monobasic (mw: 136.09 g/mol)	0.0018 M	0.0245 g
ddH ₂ O	n/a	200 mL
Total	n/a	200 mL

Alternatives:

- The Synergy H1 plate reader may be replaced by other BioTek microplate readers compatible with a red FP filter cube, including: Cytation 5, Cytation Multi-Mode, and Cytation 1. These readers all utilize the Gen5 Microplate Reader and Imager Software; however, certain assay parameters may differ by machine. Alternative FP-compatible multi-mode plate readers include: BMG LABTECH

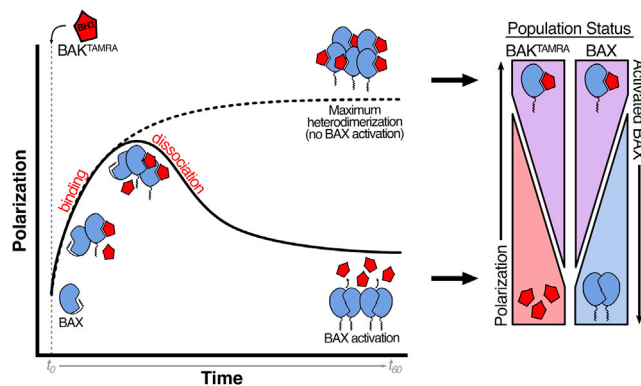


Figure 2. Schematic of BAX and BAK^{TAMRA} interactions within FLAMBE

For this assay, we use a TAMRA-labeled BAK^{BH3} peptide (BAK^{TAMRA}) and monitor changes in Polarization as a consequence of binding to BAX. As the BAX population binds BAK^{TAMRA}, Polarization increases and plateaus if the entire population is heterodimeric (dotted line). Upon BAX activation, BAK^{TAMRA} is displaced resulting in a decrease in Polarization over time as the population of unbound BAK^{TAMRA} molecules increases (solid line). This diagram is reproduced and adapted from Gelles et al. (2022).

POLARstar Omega®, PerkinElmer® VICTOR® Nivo™, and Tecan Infinite® 200 PRO. Investigators will have to consult the manufacturer's documentation to set up an analogous protocol to measure fluorescence polarization and adjust the steps within this protocol for their plate reader.

- Investigators may purchase commercially available recombinant human BAX protein, but the preferred method is to express and purify recombinant BAX protein in-house (Suzuki et al., 2000; Gelles et al., 2022).
- Unlabeled BAK-BH3 peptide (AnaSpec Cat#: AS-61616) may be purchased and conjugated to a different fluorescent label at the investigator's discretion provided the plate reader has a compatible FP cube installed or available.
- This protocol may be adapted by using alternative reporter peptides that bind BAX at distinct sites (example reporter peptides used in Pritz et al., 2017; Garner et al., 2019). We have validated the use of BAK^{TAMRA} to monitor BAX activation kinetics but investigators using alternative peptides will have to validate their assay to exclude the possibility of direct competition and properly interpret binding and dissociation patterns.
- The multi-channel pipette, 96-well PCR plate, and 96-well black polystyrene microplate can be substituted with equivalent products. While optional, using a multi-channel pipette and a PCR plate as a reagent reservoir is strongly recommended for generating optimal and reproducible results with this protocol (detailed below).

STEP-BY-STEP METHOD DETAILS

We developed a fluorescence polarization assay for monitoring BAX early-activation (abbreviated as "FLAMBE"; see Gelles et al., 2022) in which a TAMRA-labeled BAK-BH3 peptide (BAK^{TAMRA}) binds to, but does not activate, BAX (Czobotar et al., 2013; Dewson et al., 2012). Binding of BAK^{TAMRA} to BAX results in an increase in Polarization over time, reaching a plateau as the maximum number of heterodimers form (Figure 2, dotted line). In samples exhibiting BAX activation, intramolecular rearrangements within BAX are accompanied by a concomitant release of BAK^{TAMRA} and a decrease in Polarization signal over time (Figure 2, solid line). At any given time, the Polarization value represents the population fraction of BAK^{TAMRA} bound to BAX, and therefore indicates the fraction of activated BAX following dissociation (Figure 2, right). We have validated that changes in Polarization reflect the binding status of BAK^{TAMRA} and fully characterized the FLAMBE technique in Gelles et al. (2022).

This section details three types of titration experiments to monitor BAX activation, and can be used sequentially as a workflow to determine the effect of a candidate ligand on BIM^{BH3}-induced BAX

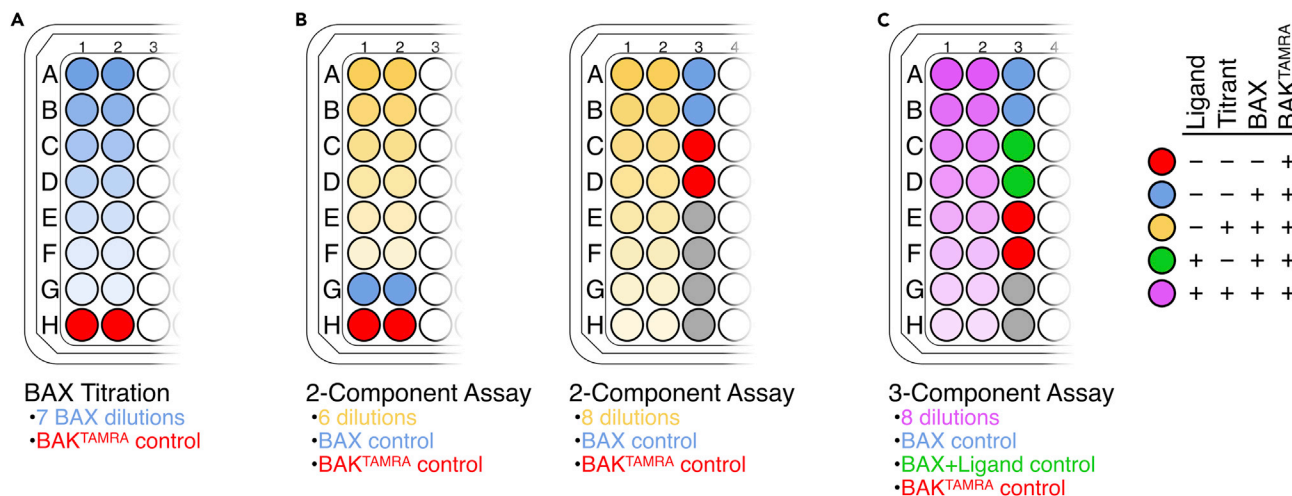


Figure 3. Model plate layouts for FLAMBE assays

(A) A BAX titration may be performed in duplicate as a 7-point dilution series (blue) and a BAK^{TAMRA} control (red).

(B) 2-component assays include BAX (blue) and a titrant (yellow). This may be set up as a 2-column (left) or 3-column (right) assay, facilitating either a 6-point or 8-point titration, respectively.

(C) 3-component assays include BAX (blue), a ligand (green), and a titrant (purple).

The Gen5 software only permits selection of consecutive wells; therefore, gray wells are designated for additional controls or empty wells that will be read during the assay.

activation. All assays must be performed in a black 96-well microplate and step-by-step details and timing for each major experiment are provided. It is recommended that experimental samples do not exceed duplicates to avoid delays in preparing replicates (see the [limitations](#) section for more details). Typical investigations will include a BAX titration and a 2- or 3-component assay (Figure 3). The experimental wells must be in consecutive columns and every experiment must include a BAK^{TAMRA} control. Investigators must also include a "0" control, which represents the "0" concentration of the titrant (commonly BAX+BAK^{TAMRA}, but may include additional components that constitute the background). To reduce pipetting error across duplicates, we recommend using a multi-channel pipette when performing the serial dilution of BAX or a titrant (Figure 4A). To capture the rapid binding kinetics of BAK^{TAMRA}, we recommend using a PCR plate (or similar) as a reagent reservoir to quickly add BAK^{TAMRA} (or other components) to all wells (Figure 4B).

Determine an appropriate BAX concentration for downstream investigations

⌚ Timing: 70–80 min

Studying BAX is a bit of a Catch-22 – sufficient protein is necessary to achieve and detect robust activation kinetics, but BAX may exhibit concentration-dependent auto-activation despite the absence of an activator. The ideal BAX concentration for FLAMBE experiments is just below the auto-activating point; this will ensure that downstream assays resolve the activation kinetics in response to activators or modulators. However, certain experiments may benefit from using BAX at a slightly activating concentration (e.g., inhibition studies). Diligent characterization of BAX concentrations within FLAMBE allows the user to calibrate downstream investigations and mitigates any variability that may arise between protein batches, concentration determination, or protein aggregation during prolonged storage at –80°C. Investigators should perform this experiment on each day of investigation using fresh aliquots of BAK^{TAMRA} and recombinant BAX (see [problem 3](#) in the [troubleshooting](#) section).

1. Keep 0.5× PBS on ice.

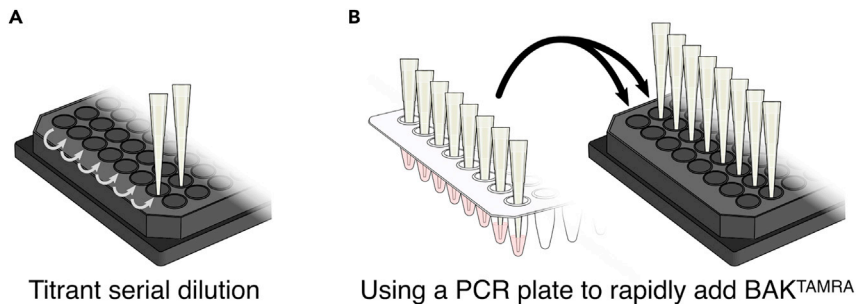


Figure 4. Setting up a FLAMBE assay

(A) We recommend investigators perform a serial dilution of the titrant in duplicate using a multi-channel pipette. The final dilution volume must be discarded to obtain the correct volume in the final dilution wells.

(B) To capture rapid binding kinetics of BAK^{TAMRA}, we recommend using a PCR plate as a reagent reservoir for quickly adding additional components and/or BAK^{TAMRA}. Since BAK^{TAMRA} is the probe, it must be added last before running the assay to capture binding kinetics.

2. Prepare assay reagents.

a. Prepare BAX.

- i. Thaw a freezer stock of BAX protein on ice. Since BAX may precipitate out of solution, we recommend centrifuging at 2,000 × g for 10 s at 4°C to pellet any large precipitates and pipetting only soluble protein.

Note: Depending on the volume of the BAX aliquots, multiple tubes may be required to obtain the appropriate volume for the titration. We recommend thawing all tubes on ice and pooling the aliquots into one tube before use.

- ii. Prepare 320 μL of a 500 nM (4×) working stock of BAX in 0.5× PBS and keep on ice.

Note: In our experience, a maximum of 125 nM (final concentration) BAX in this assay is sufficiently auto-activating such that initial binding kinetics are minimally detectable. Due to variability in protein quality or purification, investigators may find that a higher or lower maximum concentration may be more appropriate.

Note: This volume is sufficient to aliquot 150 μL into the first duplicate wells of the serial dilution with 20 μL extra to account for human/pipetting error.

b. Prepare BAK^{TAMRA}.

- i. Thaw a 2.2 μL aliquot of 0.5 mM BAK^{TAMRA} on the benchtop or at 25°C.
- ii. Prepare a 1 μM master stock of BAK^{TAMRA} by mixing 998 μL of 0.5× PBS and 2 μL of the 0.5 mM BAK^{TAMRA} freezer stock. Keep on ice and protected from light.

Note: The master stock may be stored at 4°C and used for 1–2 additional days if protected from light, but may suffer a slight decrease in peptide quality (see [problems 1](#) and [2](#) in the [troubleshooting](#) section). Investigators may reuse these stocks at their discretion.

- iii. Prepare a 200 nM (4×) working stock of BAK^{TAMRA} by mixing 560 μL of 0.5× PBS and 140 μL of the 1 μM BAK^{TAMRA} stock. Keep on ice and protected from light.

Note: Investigators will have a surplus of the BAK^{TAMRA} master stock, so for each experiment prepare the 200 nM working stock in 700 μL, which is sufficient to aliquot 25 μL per well in three columns and includes 100 μL extra to avoid pipetting errors when loading and pipetting from the PCR plate.

3. Prepare a 1.2× serial dilution of BAX in duplicate in the plate (Figure 3A).

Note: We suggest a 1.2× dilution as this will provide sufficient resolution for investigators to identify the ideal concentration of BAX for their downstream assays. While larger dilution factors may test a greater range of concentrations, the loss in resolution will likely result in users having to randomly select a concentration between those tested for downstream assays, which affects reproducibility.

- a. Aliquot 150 μL of the 4× BAX stock into duplicate wells in row A.
- b. Add 25 μL of 0.5× PBS to duplicate wells in rows B–H.
- c. Using a multi-channel pipette, serially dilute BAX in 125 μL from row A to row G (Figure 3A). Discard the final 125 μL from row G to obtain the correct final volume of 25 μL per well.

Note: Mix well by pipetting up and down several times before continuing to the next row. Avoid generating bubbles.

Note: It is recommended to confine FP assays to 7-point dilutions positioned vertically from rows A–G; row H is the BAK^{TAMRA} control (Figure 3A). In cases where investigators need to assess a larger range of BAX concentrations, 11 dilutions may be organized horizontally across the plate, with the BAK^{TAMRA} control in column 12.

- d. Using a multi-channel pipette, add 50 μL of 0.5× PBS to all wells.

△ **CRITICAL:** Investigators should initiate the FP protocol prior to the addition of BAK^{TAMRA} in the next step (see steps 12–15 of the [before you begin](#) section).

4. Using a multi-channel pipette, quickly add 25 μL of the 200 nM BAK^{TAMRA} working stock to all wells and immediately proceed to the next step.

△ **CRITICAL:** This step must be completed as quickly as possible to capture rapid binding kinetics. It is recommended to aliquot the BAK^{TAMRA} working stock in 8 wells of a PCR plate (or similar) and use a multi-channel pipette to add BAK^{TAMRA} column-by-column (Figure 4B). We recommend aliquoting 87.5 μL of BAK^{TAMRA} into 8 wells of a PCR plate to ensure sufficient excess and limit pipetting errors or delays.

Note: We have found that keeping all reagents on ice prior to plate setup minimizes molecular interactions among reagents prior to running the assay. This ensures detection of initial binding kinetics.

Note: The final concentration of BAK^{TAMRA} per well is 50 nM. The final BAX concentration range for this described setup is 42–125 nM.

Note: The final volume per well is 100 μL.

5. Load the plate onto the tray and initiate the assay by clicking "OK". The assay will run for 1 h. Following assay completion, the plate will be ejected. Save the exported file. Visualize kinetic data in GraphPad Prism, Microsoft Excel, or a similar program to determine an appropriate concentration of BAX with which to proceed (see [expected outcomes](#) section and Figure 5A for more details).

▮ **Pause point:** Investigators should determine their ideal concentration of BAX and proceed to the next section with the same aliquots of reagents. The identified concentration may be used for future assays, but we strongly recommend repeating this step periodically or with

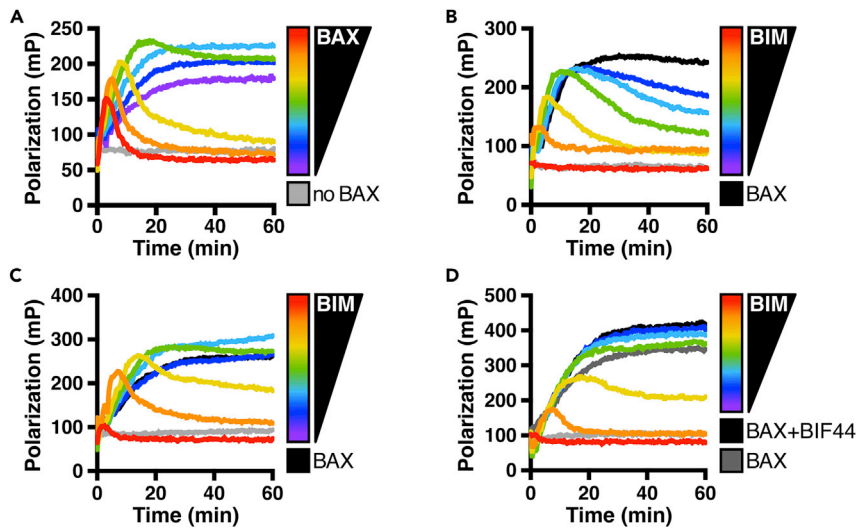


Figure 5. Experimental kinetic FLAMBE data

(A) BAK^{TAMRA} (50 nM) was added to BAX (42–125 nM) and fluorescence polarization was measured. Stable BAX:BAK^{TAMRA} heterodimer formation is observed at lower concentrations (purple and blue data). The threshold concentration for BAX auto-activation is identified in green as the lowest concentration that exhibits a decrease in Polarization i.e., BAK^{TAMRA} displacement. Higher concentrations result in faster kinetics of BAK^{TAMRA} displacement (yellow through red data). Data are reproduced from Gelles et al. (2022).

(B) BAK^{TAMRA} (50 nM) was added to BAX (60 nM) in the presence of BIM^{BH3} (BIM, 0.08–2.5 μM) and fluorescence polarization was measured. Gray line represents BAK^{TAMRA} control. The BAX control (black data) is weakly auto-activating, resulting in a step-wise trend in displacement of BAK^{TAMRA} as BAX is activated by BIM^{BH3}.

(C) Similar experiment as in (B). BAK^{TAMRA} (50 nM) was added to BAX (40 nM) in the presence of BIM^{BH3} (BIM, 0.04–1.2 μM) and fluorescence polarization was measured. Gray line represents BAK^{TAMRA} control. The non-activating concentration of BAX (black data) reveals that low concentrations of BIM^{BH3} result in stable binding to the BAK:BAK^{TAMRA} heterodimer and an increase in Polarization (blue data). The threshold BIM^{BH3} concentration for activating BAX is identified in green and additional concentrations of BIM^{BH3} increase activation kinetics (yellow through red data).

(D) FP assay in which BAK^{TAMRA} (50 nM) was added to BAX (40 nM) in the presence of BIM^{BH3} (BIM, 0.04–1.2 μM) and BIF44 (250 μM). Binding of BIF44 to BAX reveals an increase in Polarization compared to the BAX control (dark gray compared to black data). The addition of BIM^{BH3} reveals a step-wise progression of BAX activation kinetics, demonstrating that the BAX+BIF44 condition was at the threshold concentration for activation.

each new batch of BAX for reproducibility and quality control purposes. It is not recommended to reuse these reagents for future experiments conducted on a different day.

Perform a BAX activation assay using a BIM^{BH3} titration

⌚ Timing: 70–80 min

The following steps describe the protocol for measuring and characterizing trends of BIM^{BH3}-induced BAX activation. BIM is the predominant BCL-2 family protein responsible for activating BAX in cells, converging a myriad of stress and pro-apoptotic signal pathways onto BAX-mediated apoptosis (Sarosiek et al., 2013). BAX exhibits multiple conformers following activation by BIM and therefore investigators are likely to test their ligand of interest within the context of BIM-activated BAX (additional details regarding BIM triggering and the BAX activation continuum are discussed in Gelles et al., 2022). We describe using a BIM^{BH3} peptide due to its commercial availability, stability, and potent activation of BAX; alternative BIM reagents (e.g., recombinant BIM protein, stapled BIM SAHB peptide) may be substituted but require more specialization to produce.

6. Keep 0.5× PBS on ice.

7. Prepare assay reagents.

a. Prepare BAX.

- i. In 0.5× PBS, prepare 450 μL of a BAX working stock that is 4× the non-activating concentration of BAX identified in the previous experiment and keep on ice.

Note: For consistency, we strongly recommend using BAX protein from the freezer stock that was thawed and pooled for the previous experiment. Alternatively, investigators may thaw a fresh tube of BAX, however, slight changes in signal and optimal concentration may occur due to protein variability.

Note: This volume is sufficient to aliquot 25 μL per well with 100 μL extra to account for human/pipetting error.

b. Prepare BAK^{TAMRA}.

- i. From the 1 μM BAK^{TAMRA} master stock prepared in step 2b (or prepared fresh), prepare 700 μL of a 200 nM (4×) working stock of BAK^{TAMRA} in 0.5× PBS. Keep on ice and protected from light.

Note: The master stock may be stored at 4°C and used for 1–2 additional days if protected from light but may suffer a slight decrease in peptide quality (see [problems 1](#) and [2](#) in the [troubleshooting](#) section). Investigators may reuse these stocks at their discretion.

Note: Investigators will have a surplus of BAK^{TAMRA} master stock, so for each experiment prepare the 200 nM working stock in 700 μL, which is sufficient to aliquot 25 μL per well in three columns and includes 100 μL extra to avoid pipetting errors when loading and pipetting from the PCR plate.

8. Prepare a 2× serial dilution of BIM^{BH3} in the plate as duplicates. We recommend starting the range at 30–50-fold molar excess of BAX and diluting down.

- a. Prepare 120 μL of a 4× stock of BIM^{BH3} in 0.5× PBS. Aliquot 50 μL into duplicate wells in row A.
- b. Add 25 μL of 0.5× PBS to all other titrant wells (rows B–F, see [Figure 3B](#), left panel).
- c. Using a multi-channel pipette, serially dilute BIM^{BH3} in 25 μL from row A to F. Discard the final 25 μL from row F to obtain the correct final volume of 25 μL.

Note: Mix well by pipetting up and down several times before continuing to the next row. Avoid generating bubbles.

Note: This protocol describes a 6-point BIM^{BH3} titration ([Figure 3B](#), left panel). For a wider titration, this setup may be expanded to an 8-point titration with a third column used for controls ([Figure 3B](#), right panel). Similarly, the dilution factor may be adjusted to increase resolution of the BIM^{BH3} concentrations at the discretion of the investigator.

9. Using a multi-channel pipette, add 25 μL of 0.5× PBS to wells in rows A–F, 50 μL to row G, and 75 μL to row H.

Note: Rows A–F will contain the BIM^{BH3} titration, row G will contain the BAX+BAK^{TAMRA} control, and row H will contain the BAK^{TAMRA} control (see [Figure 3B](#), left panel).

10. Aliquot the 4× BAX stock in 7 wells of a PCR plate (or similar). Using a multi-channel pipet, add 25 μL of BAX to rows A–G. Each well will now contain 75 μL.

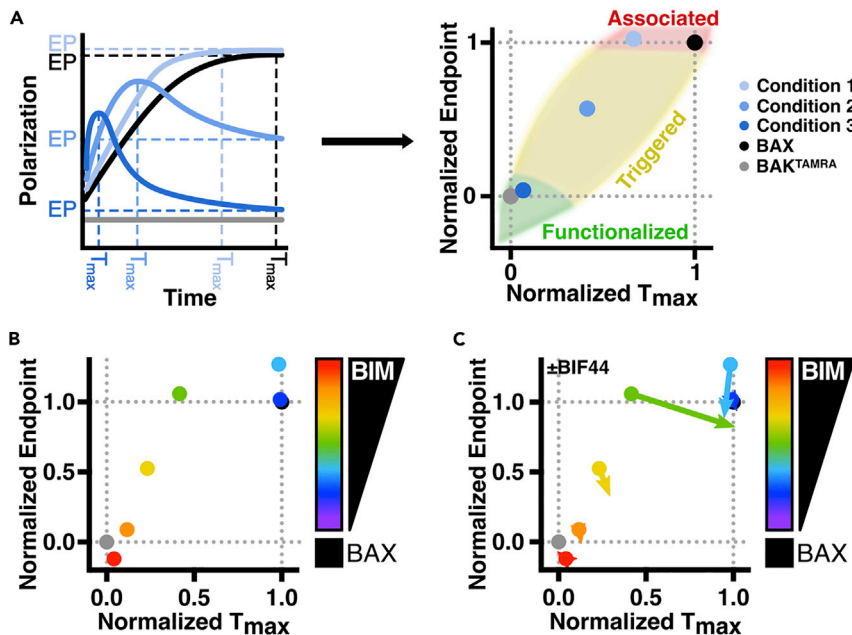


Figure 6. FLAMBE data parameterization and treatment comparison

(A) Schematic depicting FLAMBE data parameterization. Left: Blue lines represent BAX treatment conditions. Endpoint Polarization signal (EP) and time-to-maximum signal (T_{max}) are extracted from kinetic data and normalized to BAX and BAK^{TAMRA} controls (illustrated in black and gray, respectively). Right: Theoretical data may be positioned into defined regions indicative of "associated", "triggered", or "functionalized" BAX responding to treatment (further defined in Gelles et al., 2022). Panels are reproduced and adapted from Gelles et al. (2022).
 (B) Parameterization of data in Figure 5C.
 (C) Parameterized data in Figure 5C compared to parameterized data in Figure 5D. Arrows depict a shift due to the presence of BIF44.

△ **CRITICAL:** Investigators should initiate the FP protocol prior to the addition of BAK^{TAMRA} in the next step (see steps 12–15 of the before you begin section).

- Using a multi-channel pipette, add 25 μ L of BAK^{TAMRA} column-wise to all wells. Immediately proceed to the next step.

△ **CRITICAL:** This step must be completed as quickly as possible to capture rapid binding kinetics. It is recommended to aliquot the BAK^{TAMRA} working stock in 8 wells of a PCR plate (or similar) and use a multi-channel pipette to add BAK^{TAMRA} column-by-column (Figure 4B). We recommend aliquoting 87.5 μ L of BAK^{TAMRA} into the PCR plate to ensure sufficient excess and limit pipetting errors or delays.

Note: The final concentration of BAK^{TAMRA} is 50 nM.

Note: The final volume in all wells is 100 μ L.

- Load the plate onto the tray and initiate the assay by clicking "OK". The assay will run for 1 h. Following assay completion, the plate will be ejected. Save the exported file. Visualize kinetic data in GraphPad Prism, Microsoft Excel, or a similar program (see the expected outcomes section and Figures 6B and 6C for more details; for additional data analysis see the quantification and statistical analysis section). Proceed to the next section if applicable for your experimental design.

Note: If applicable, it is recommended to perform an additional analogous experiment using a candidate ligand titration to reveal the effect, if any, of the ligand on BAX (this experiment may also identify an appropriate ligand concentration for the experiment in the next section). While we have detailed a BIM^{BH3} titration, the steps above may be adapted for a ligand titration experiment.

Perform a comparative activation assay using a candidate ligand and BIM-activated BAX

⌚ Timing: 70–80 min

The following steps describe the protocol for setting up a 3-component assay to study the effect of a ligand (used at a constant concentration) on dose-dependent BIM^{BH3}-induced activation of BAX (Figure 3C). The ideal concentration of the ligand should be determined by performing a ligand titration experiment analogous to the steps in the preceding section. While we describe the single-dose ligand experiment below, the steps may be adapted for the reciprocal experiment using a single dose of BIM^{BH3} and a titration of the ligand of interest. Importantly, for comparative assays we strongly recommend using the same dilution factor and titration point for each experiment.

13. Keep 0.5× PBS on ice.
14. Prepare assay reagents.
 - a. Prepare BAX.
 - i. In 0.5× PBS, prepare 700 μL of a BAX working stock that is 4× the non-activating concentration of BAX identified in the previous experiment and keep on ice.

Note: For consistency, we strongly recommend using BAX protein from the freezer stock that was thawed for the previous experiment. Alternatively, investigators may thaw a fresh tube of BAX, however, slight changes in signal and optimal concentration may occur due to protein variability.

Note: This volume is sufficient to aliquot 25 μL per well with 100 μL extra to account for human/pipetting error.

- b. Prepare BAK^{TAMRA}.
 - i. From the 1 μM BAK^{TAMRA} master stock prepared in step 2b (or prepared fresh), prepare 700 μL of a 200 nM (4×) working stock of BAK^{TAMRA} in 0.5× PBS. Keep on ice and protected from light.

Note: The master stock may be stored at 4°C and used for 1–2 additional days if protected from light, but may suffer a slight decrease in peptide quality (see [problems 1](#) and [2](#) in the [troubleshooting](#) section). Investigators may reuse these stocks at their discretion.

Note: Investigators will have a surplus of the BAK^{TAMRA} master stock, so for each experiment prepare the 200 nM working stock in 700 μL, which is sufficient to aliquot 25 μL per well in three columns and includes 100 μL extra to avoid pipetting errors when loading and pipetting from the PCR plate.

- c. Prepare the ligand of interest.
 - i. Prepare 700 μL of a 4× candidate ligand working stock in 0.5× PBS.

Note: The concentration of the ligand working stock should be 4 times the concentration of an effective dose identified in an FP assay (see previous section) or a BAX functional assay (e.g., LUV permeabilization assay).

15. Prepare a 2× serial dilution of BIM^{BH3} in the plate as duplicates. We recommend starting the range at 30–50-fold molar excess of BAX and diluting down.
 - a. Prepare 120 μL of a 4× stock of BIM^{BH3} in 0.5× PBS. Aliquot 50 μL into duplicate wells in row A.
 - b. Add 25 μL of 0.5× PBS to all other titrant wells (rows B–H, see [Figure 3C](#)).
 - c. Using a multi-channel pipette, serially dilute BIM^{BH3} in 25 μL from row A to H. Discard the final 25 μL from row H to obtain the correct final volume of 25 μL.

Note: Mix well by pipetting up and down several times before continuing to the next row. Avoid generating bubbles.

Note: This protocol describes an 8-point BIM^{BH3} titration ([Figure 3C](#), purple wells). The dilution factor may be adjusted to increase resolution of the BIM concentrations at the investigator's discretion.

16. Set up control wells.
 - a. Set up the BAX controls.
 - i. In two consecutive wells of the third column, add 50 μL of 0.5× PBS followed by 25 μL of the 4× BAX working stock and 25 μL of BAK^{TAMRA}. These wells will be the BAX+BAK^{TAMRA} control ([Figure 3C](#), blue wells).
 - ii. In two consecutive wells of the third column, add 25 μL of 0.5× PBS followed by 25 μL of the 4× BAX working stock, add 25 μL of the 4× ligand working stock, and 25 μL of BAK^{TAMRA}. These wells will be the BAX+Ligand+BAK^{TAMRA} control ([Figure 3C](#), green wells).
 - b. Set up the BAK^{TAMRA} control.
 - i. In two consecutive wells of the third column, add 75 μL of 0.5× PBS followed by 25 μL of the 200 nM BAK^{TAMRA} stock ([Figure 3C](#), red wells).

Note: The 3-component plate layout includes two additional wells in which investigators may choose to also include a ligand control containing 50 μL of 0.5× PBS, 25 μL of the 4× ligand working stock, and 25 μL of BAK^{TAMRA} ([Figure 3C](#), gray wells). The Gen5 software requires selection of consecutive wells; therefore, these wells may be left empty if no additional controls are required.

Note: Since the conditions for the controls were selected based on plateauing concentrations in previous experiments, kinetics for the controls are less important, than that of the samples. To ensure the capture of initial binding kinetics, we recommend adding BAK^{TAMRA} to the control wells prior to setting up sample wells to minimize the time between the addition of BAK^{TAMRA} to sample wells and initiation of the assay.

17. Divide the remaining 650 μL of 4× ligand working stock in 8 wells of a PCR plate. Using a multi-channel pipette, aliquot 25 μL per well in all titrant wells ([Figure 3C](#), purple wells in columns 1 and 2).
18. Divide the remaining 600 μL of 4× BAX working stock in 8 wells of a PCR plate. Using a multi-channel pipette, add 25 μL per well in all titrant wells ([Figure 3C](#), purple wells in columns 1 and 2).

△ CRITICAL: Investigators should initiate the FP protocol prior to the addition of BAK^{TAMRA} in the next step (see steps 12–15 of the [before you begin](#) section).

19. Divide the remaining 550 μL of the 200 nM BAK^{TAMRA} working stock in 8 wells of a PCR plate. Using a multi-channel pipette, add 25 μL of BAK^{TAMRA} to all titrant wells ([Figure 3C](#), purple wells). Immediately proceed to the next step.

△ **CRITICAL:** This step must be completed as quickly as possible to capture rapid binding kinetics. It is recommended to aliquot the BAK^{TAMRA} working stock in 8 wells of a PCR plate (or similar) and use a multi-channel pipette to add BAK^{TAMRA} column-by-column (Figure 4B). We recommend aliquoting 68.75 μL of BAK^{TAMRA} into the PCR plate to ensure sufficient excess and limit pipetting errors or delays.

Note: The final concentration of BAK^{TAMRA} is 50 nM.

Note: The final volume in all wells is 100 μL.

20. Immediately load the plate onto the tray and initiate the assay by clicking "OK". The assay will run for 1 h. Following assay completion, the plate will be ejected. Save the exported file. Visualize kinetic data in GraphPad Prism, Microsoft Excel, or a similar program (see the [expected outcomes](#) section and Figure 5D for more details; for additional data analysis see the [quantification and statistical analysis](#) section).

EXPECTED OUTCOMES

This section describes methods to visualize and interpret data generated in the FLAMBE workflow. The steps included in this section are not specific to a BioTek Synergy H1 Hybrid Multi-Mode Plate Reader and may be implemented downstream of any FP-compatible microplate reader.

The Gen5 software automatically calculates Polarization during data analysis so investigators using alternative plate readers may need to manually derive Polarization from raw intensity (*I*) values according to the following equation:

$$P_t = \frac{I_{||t} - I_{\perp t}}{I_{||t} + I_{\perp t}}$$

where: P_t is the Polarization value for a sample at a given time point, t ; $I_{||t}$ is the parallel intensity value at time point t ; and $I_{\perp t}$ is the perpendicular intensity at time point t . Polarization is a unitless metric but is conventionally expressed as milliPolarization (*mP*) units.

An initial BAX titration experiment is required to identify the ideal concentration for downstream BAX activation studies. Investigators must reconcile two opposing considerations regarding BAX biology: (i) BAX may auto-activate at high concentrations; and paradoxically, (ii) upon activation, BAX requires an adequate concentration to sufficiently perpetuate within the population. The first point is demonstrated in Figure 5A, where the BAX threshold for auto-activation was observed (green data); concentrations exhibiting auto-activation are generally not appropriate to select for downstream investigations but may be relevant to inhibition studies (Figure 5A, red through green data). In contrast, lower range concentrations achieved stable plateaus and exhibited a step-wise increase in Polarization values, which is expected as more BAX in the sample forms heterodimers with BAK^{TAMRA} (Figure 5A, blue and purple lines). The second point is important for selecting which of the stably-binding concentrations is most appropriate. Choosing a concentration too close to the threshold risks BAX stochastically auto-activating in downstream assays while choosing too low a concentration risks insufficient population responses following BAX activation. The ideal concentration is just below threshold, which will ensure adequate BAX concentrations for downstream activation studies (in Figure 5A we would select the concentration of the light blue data). Additionally, this will ensure that the ratio of BAX:BAK^{TAMRA} will generate data with sufficient resolution to observe activation kinetics.

In a cell, BIM is the predominant activator of BAX. We employed a BIM-BH3 peptide (BIM^{BH3}) to study BAX activation in this assay as it has been shown to target a distinct "trigger" site, which is

distal to the predicted BAK^{TAMRA} binding site (Gavathiotis et al., 2008). Investigators utilizing FLAMBE to study BAX activation should verify the binding site of a candidate ligand on BAX to accurately interpret kinetic FP data (see the [limitations](#) section). Activation studies with BIM^{BH3} may be used to determine the sensitivity of BAX to activate and are typically conducted as BIM^{BH3} titrations; these data may also be informative for more specific synergistic experiments (an example of such an experiment is shown in Figures 5H and 5I of Gelles et al., 2022). Trends in BIM^{BH3} titration experiments are dependent on the concentration of BAX and may exhibit different patterns (Figures 5B and 5C). If the BAX concentration is at or near the auto-activation threshold (slight downward trend in Figure 5B, black data), investigators will likely observe a step-wise progression of dissociation kinetics as the population is proportionally activated by the amount of BIM^{BH3} (Figure 5B). If the selected concentration of BAX is far below threshold, investigators may observe that low BIM^{BH3} doses exhibit increased Polarization and faster binding kinetics compared to the BAX control (Figure 5C, light blue data). This increase in Polarization is indicative of heterotrimerization – stable binding of BIM^{BH3} to the BAK:BAK^{TAMRA} heterodimer without BAX activation (Tsai et al., 2015). While either outcome is valid, investigators should note how the "tipping point" of BIM activation is affected by the selection of BAX concentration. The activation profile in Figure 5C may be preferable as it will indicate non-activating concentrations of BIM^{BH3}, which may be useful for subsequent assays. Of note, similar trends and considerations may also apply for ligand titration experiments.

3-component assays may be used to study the synergistic response of BAX to BIM and a binding ligand (here, "ligand" is not limited to being a small molecule and we have tested a diverse repertoire of BAX modulators including peptides, lipids, and detergents; see Gelles et al., 2022). To illustrate this experimental workflow, we have included data for a duplicate BIM^{BH3} titration in the presence of a small molecule BAX activator, BIF44 (Figure 5D) (Pritz et al., 2017). A common feature of these experiments is a global increase in Polarization caused by the ligand binding to the BAX:BAK^{TAMRA} heterodimer (Figure 5D, "BAX+BIF44" compared to "BAX"). While differences between assays may be qualitatively assessed, a more direct method may be necessary to properly compare the data and infer conclusions (compare Figures 5C and 5D). We developed a method to parameterize kinetic FLAMBE data and visualize changes when two experiments are compared (see the [quantification and statistical analysis](#) section). Of note, investigators should also perform the reciprocal experiment using a titration of ligand in the presence of a single concentration of BIM^{BH3}. This may be compared to an identical ligand titration experiment conducted in the absence of BIM^{BH3}.

QUANTIFICATION AND STATISTICAL ANALYSIS

This section will describe how to parameterize FLAMBE data for visualization and comparative analyses. Kinetic graphs are the preferred method to report FLAMBE data and therefore parameterization is optional. However, parameterized data benefits from being more succinct, making it suited for certain applications. The following steps will detail how investigators may use Microsoft Excel or GraphPad Prism to parameterize their data but may be adapted for other software. Alternatively, investigators may utilize our included "FLAMBE_Parameterization" Excel template (Data S1), which is set up to automatically parameterize metrics from imported raw data and may simplify the process for users who are less familiar with Excel formulas.

Parameterize FLAMBE kinetic data

FLAMBE captures the kinetics of BAX activation, which is more data-rich and informative than endpoint observations but may also be more difficult to interpret or discuss. To distill kinetic FLAMBE data, we selected a dual-metric parameterization strategy: endpoint Polarization (EP) and the time point of maximum Polarization (T_{max}) (Figure 6A, left panel). Based on coupled functional assays, we have demonstrated that these parameters allow investigators to make accurate inferences as to the functional state of activated BAX at the end of the assay (Figure 6A, right panel) (Gelles et al., 2022).

1. Calculate the mean for replicate time points.
 - a. We suggest averaging replicates prior to parameterization to smooth the data. Even miniscule deviations between replicates or data noise may substantially affect the determination of T_{\max} (and, to a lesser extent, EP) resulting in wide T_{\max} ranges. Averaging the replicates prior to parameterization will generate more reliable metrics (see [problem 6](#) in the [troubleshooting](#) section for additional advice on parameterizing noisy Polarization data).
 - i. In Excel, this is accomplished using the MEAN function.
 - ii. In Prism, analyze the data table using "Row Statistics" and selecting "Compute the mean, median, etc. for each data set column" with "means" and "SD, N" selected in the drop-down menus.
 - iii. If using the FLAMBE_Parameterization template, data pasted into the "Raw_Data" sheet will automatically be averaged in the "Averages" sheet.
2. Determine EP for samples.
 - a. Endpoint Polarization (EP) is simply the Polarization values for samples at the completion of the FP assay. Due to the dynamic binding and displacement of $\text{BAK}^{\text{TAMRA}}$ during BAX activation, EP alone is not sufficiently informative and should not be used as the sole metric to summarize FLAMBE data.
 - i. EP values may be taken directly from the final read of the averaged data from step 1. Alternatively, investigators may average the last 3–5 data points to minimize the influence of noise.
 - ii. If using the FLAMBE_Parameterization template, EP is automatically extracted from the final time point, regardless of the assay duration, and is populated in the "Parameterization" sheet.
 - b. The EP metric may provide insights into the percentage of the BAX population that has been activated. For example, the yellow line in [Figure 5D](#) flattens, which indicates that approximately 50%–60% of the BAX population released $\text{BAK}^{\text{TAMRA}}$ but the activation stimulus was insufficient to activate the entire population. At t_{60} some conditions may still be resolving, but extending beyond this time negligibly impacts the data.
 - c. Generally, EP values near the $\text{BAK}^{\text{TAMRA}}$ control indicate activation while values near the BAX control indicate association, but no BAX activation. EP values may be greater than the BAX control if the titrant binds to the $\text{BAX}:\text{BAK}^{\text{TAMRA}}$ heterodimer and increases the molecular volume – these data are still interpreted as association events with no activation.
3. Determine T_{\max} for samples.
 - a. Time of maximum Polarization (T_{\max}) is the time point with the highest Polarization value for a given sample. First, identify the maximum Polarization value for each sample from the averaged data in step 1 and then identify the time point at which that maximum occurred.
 - i. In Excel, determine the maximum Polarization for each sample using the MAX function. To identify the corresponding time point, use either the XLOOKUP function (if available in your version of Excel) or a nested formula with INDEX and MATCH (this two-part formula first identifies the row of the spreadsheet and then may retrieve a cell in the same row but different column).
 - ii. Graphpad Prism does not have an easy method to accomplish this, but there is a multi-step workaround. (i) Analyze the data table using "Normalization", setting the 0% as the "smallest value in each data set" and the 100% as the "largest value in each data set", and present results as fractions. (ii) Next, analyze the normalized data table using "Transform" and, in the next window, create a user-defined Y function with the equation " $Y=\text{if}(Y=1,X,0)$ ". (iii) Finally, analyze the transformed data table using "Descriptive Statistics" with only the "Minimum and maximum, range" option selected – the "maximum" values listed are the T_{\max} for each sample.
 - iii. If using the FLAMBE_Parameterization template, the maximum Polarization and T_{\max} metrics are automatically identified and provided in the "Parameterization" sheet.
 - b. For non-activating BAX samples, the T_{\max} will correlate with the EP as the population forms heterodimers and Polarization signal plateaus (see [Figure 6A](#), left panel, "Condition 1"). In

activating conditions, the T_{\max} will represent the time at which the rate of $\text{BAK}^{\text{TAMRA}}$ dissociation outpaces binding. Therefore, low T_{\max} values indicate BAX activation since $\text{BAK}^{\text{TAMRA}}$ dissociation must have occurred if the maximum Polarization was measured early in the assay (see [Figure 6A](#), left panel, "Conditions 2 & 3").

Note: T_{\max} for samples exhibiting very rapid BAX activation may not be detected by the time the measurements begin (for example, the red data in [Figure 5B](#)). Since the data remains fairly constant during the assay, identification of a T_{\max} will be unreliable and may be skewed by noise within the kinetic data. For these samples, consider the T_{\max} to be 0.

△ CRITICAL: T_{\max} for $\text{BAK}^{\text{TAMRA}}$ is always set to 0.

4. Normalize EP and T_{\max} to the controls.
 - a. Parameterized metrics will be normalized against the $\text{BAK}^{\text{TAMRA}}$ control (set to 0) and the BAX control (set to 1) ([Figure 6B](#), which is the parameterized data from [Figure 5C](#)). Using this method, data from multiple experiments with similar conditions may be compared on the same plot ([Figure 6C](#), which is the comparison of parameterized data from [Figures 5C](#) and [5D](#)).
 - i. In Excel, parameterized metrics may be normalized using the formula " $(X - X_{\text{BAK}}) / (X_{\text{BAX}} - X_{\text{BAK}})$ ", where: X is the metric (either EP or T_{\max}); X_{BAK} is the metric from the $\text{BAK}^{\text{TAMRA}}$ control; and X_{BAX} is the metric from the BAX control.
 - ii. In Prism, generate a new XY data table with raw EP and T_{\max} values as separate Y groups. Importantly, the first row must be the $\text{BAK}^{\text{TAMRA}}$ control and the last row must be the BAX control being normalized against. Analyze the table using "Normalize" with 0% defined as "first value in each data set" and 100% defined as "last value in each data set" and presenting results as fractions.
 - iii. If using the FLAMBE_Parameterization template, the normalized metrics are automatically calculated and provided in the "Parameterization" sheet. Additionally, users may supply parameterized metrics for two similar experiments in the "BeforeAndAfter" sheet to generate a comparison graph.
 - b. While Normalized EP may be a negative value (for samples with robust activation and EP values below $\text{BAK}^{\text{TAMRA}}$), normalizing T_{\max} should never result in a negative value.
 - c. Occasionally, experiments should not be normalized if there is no consistent control to normalize samples to. As an example, testing BIM sensitivity across BAX mutants cannot be normalized because each mutant is compared to its own "no activation" condition (such an experiment is shown in [Figure 4K](#) of [Gelles et al., 2022](#)). These experiments may still be compared and visualized using the raw EP and T_{\max} values.

LIMITATIONS

The FLAMBE technique is a useful tool to study BAX activation in real-time. However, this method is accompanied by certain limitations.

The rapid kinetics of binding and release of $\text{BAK}^{\text{TAMRA}}$ from BAX restricts the number of confident replicates to duplicates, as setup of additional replicates causes a divergence in the read time for any given set of replicates, thus obfuscating kinetic trends. More rapid measurements allow for richer kinetic data collection but reduces the throughput of the assay. Due to this limitation, we suggest investigators increase the number of replicated experiments to confidently determine reproducibility.

$\text{BAK}^{\text{TAMRA}}$ is predicted to bind to the BAX BC groove and is released following activation-induced BAX conformational changes (e.g., in response to triggering by BIM). Therefore, investigating agents that target the BAX BC groove (e.g., BID) may confound data interpretation due to competition-mediated $\text{BAK}^{\text{TAMRA}}$ dissociation, which will be conflated with activation-induced $\text{BAK}^{\text{TAMRA}}$

dissociation. Additionally, the unstructured nature of the BAK^{TAMRA} peptide may confer binding flexibility and affinity to BAX. A peptide probe exhibiting secondary structure may alter the pattern of kinetic data in FLAMBE. The unstructured nature of BAK^{TAMRA} may also promote interactions with a candidate ligand – typically observed as an increase in Polarization compared to the BAX control (Figure 5D, compare "BAX" and "BAX+BIF44" lines). If the interaction is too strong, BAX activation trends may be obfuscated and investigators may either have to select an alternative labeled peptide or select a different method to study their ligand of interest.

This protocol provides instructions for carrying out FLAMBE assays in 0.5× PBS. Alternative buffers may be used for compatibility with downstream assays (e.g., LUV permeabilization assays) or to aid in folding of peptides. However, differences between buffers and salt concentration may affect the behavior of peptides in solution as well as interactions with BAX, and this will change the appearance of kinetic data.

FLAMBE observations represent a surrogate readout for activation-induced intramolecular rearrangements in BAX that promote release of BAK^{TAMRA}. While data interpretations are based in our understanding of BAX activation biology, the precise mechanism of trigger-induced BAK^{TAMRA} release remains unknown. Additionally, since FLAMBE is a membrane-free system, general conclusions about ligand-induced modulation of BAX activation should not be made solely from FLAMBE data. Observations made with FLAMBE should be accompanied by downstream functional assays (e.g., size-exclusion chromatography, membrane permeabilization assessments) to interrogate and characterize late-stage steps of the BAX activation continuum (for more details see Gelles et al., 2022).

TROUBLESHOOTING

Problem 1

The BAK^{TAMRA} Polarization signal increases during the assay or has increased from one experiment to another.

Potential solution

An increase in BAK^{TAMRA} signal likely indicates decreased peptide quality stemming from either prolonged light exposure or peptide aggregation from prolonged incubation at temperatures above 4°C (counterintuitively, both a lower fluorescent signal and probe aggregation will result in higher Polarization). Since the measured Polarization is the weighted average of every BAK^{TAMRA} molecule's Polarization, even minority subpopulations may affect the observed values. We recommend storing and preparing BAK^{TAMRA} in amber tubes to limit exposure to light (see step 2 of the [before you begin](#) section). Additionally, BAK^{TAMRA} working solutions should be made fresh from 0.5 mM master stocks on the day of the experiment and kept on ice or at 4°C, protected from light, for the duration of experimentation. To test if BAK^{TAMRA} is aggregating, investigators may compare Polarization values for BAK^{TAMRA} alone or with low concentration detergent (e.g., 0.01% Triton X-100), which would reveal if Polarization was increased due to aggregates in the population.

Problem 2

The Polarization endpoint for some of the experimental conditions are below that of the BAK^{TAMRA} control.

Potential solution

This is expected and likely due to a minority subpopulation of BAK^{TAMRA} aggregates in the stock. While the aggregates remain in the BAK^{TAMRA} control (and slightly raise the "background" Polarization value), the aggregates may separate during the process of binding and release from BAX, particularly in samples exhibiting robust activation kinetics (Figure 5A, red data). This observation does not affect data analysis or interpretation and is why normalized EP values may be negative

values. Importantly, modifications to this assay (e.g., buffer composition, alternative labeled probe) may eliminate this phenomenon.

Problem 3

The BAX control is auto-activating at a concentration at which it was stable on a previous day.

Potential solution

This is relatively common and may be due to a few reasons, including: (i) lot-to-lot variability of the recombinant protein; (ii) loss of stability due to prolonged storage; and (iii) concentration-dependent auto-activation. If the selected BAX concentration was on the cusp of exhibiting auto-activation, it is not improbable that the same concentration could stochastically exhibit activation in a subsequent assay (further discussed in the [expected outcomes](#) section). It is strongly recommended that a BAX titration is performed prior to any subsequent experimentation on a given day to confirm the selection of a non-activating concentration of BAX. Additionally, we recommend that for a given day of experimentation, investigators pool fresh aliquots of BAX to be used that same day.

Problem 4

Trends between identical BIM titration experiments are divergent even though the same concentration of BAX was used.

Potential solution

This is due to the inherent biology of BAX – conformational changes promote dimerization and multimerization resulting in activation. Prolonged storage of BAX is prone to multimerization and precipitation, which may alter the concentration of soluble protein and subsequently the final concentration of BAX within the assay. To decrease the likelihood of this occurring, it is recommended to store master stocks of BAX at a concentration of 10–30 μM (see step 1 of the [before you begin](#) section).

Problem 5

BAX activation profiles in response to BIM or a candidate ligand are meager.

Potential solution

This may occur if the concentration selected from the initial BAX titration was too low and there is not enough BAX to sufficiently activate the population in response to an activator (this consideration is discussed in the [expected outcomes](#) section). This may be rectified by revisiting the BAX titration experiment and selecting a higher concentration of BAX that forms stable heterodimers with $\text{BAK}^{\text{TAMRA}}$.

Problem 6

The kinetic data is noisy and is affecting data parameterization.

Potential solution

If the kinetic data trends are noisy, this may affect the EP and T_{max} values retrieved (for example, a spike in a datapoint for the BAX control may result in a T_{max} that does not properly reflect the kinetic trend). To resolve this issue, investigators may apply a smoothing function to the kinetic data (such as the Analysis option within Graphpad Prism). Alternatively, investigators may average the final 3–5 data points to minimize noise when obtaining EP values. Importantly, these solutions are non-disruptive and should not substantially alter the data or interpretations.

Problem 7

The kinetic curves start at a high Polarization value and decrease over time – i.e., no initial increases in Polarization due to binding are captured.

Potential solution

This is likely due to prolonged delay in loading the plate into the plate reader and commencing the assay. To capture any rapid initial binding kinetics, we recommend minimizing the time between adding BAK^{TAMRA} to experimental wells and running the assay. This time is also markedly reduced by launching the FP protocol on Gen5 beforehand (see steps 12–15 of the [before you begin](#) section).

Problem 8

The replicates are divergent and introduce large error bars in kinetic data.

Potential solution

This will occur if substantial time elapses between the addition of BAK^{TAMRA} to the first and second replicates. To reduce delays between setting up replicates, we recommend restricting experimental replicates to duplicates and using a PCR plate as a reservoir to quickly add BAK^{TAMRA} to replicate wells (see [Figures 3 and 4](#); see step 4 of the [step-by-step method details](#) section). Additionally, increasing the excess BAK^{TAMRA} in each well of the PCR plate before loading will avoid delays due to pipetting errors. This will afford users reduced lag time in reads between replicates and should reduce any deviation between replicates.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jerry Edward Chipuk (jerry.chipuk@mssm.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101252>.

ACKNOWLEDGMENTS

This work was supported by: NIH grants R01 CA259110 (J.E.C.) and R01 CA206005 (J.E.C.), the JJR Foundation, the William A. Spivak Fund, the Fridolin Charitable Trust, an American Cancer Society Research Scholar Award, a Leukemia & Lymphoma Society Career Development Award, and an Irma T. Hirschl/Monique Weill-Caulier Trust Research Award. This work was also supported in part by two research grants (5FY1174 and 1FY13416) from the March of Dimes Foundation, a Collaborative Pilot Award from the Melanoma Research Alliance, a Developmental Research Pilot Project Program within the Department of Oncological Sciences at the Icahn School of Medicine at Mount Sinai, and the Tisch Cancer Institute Cancer Center Support Grant (P30 CA196521). The authors wish to thank Drs. Thibaud T. Renault and Mark P. A. Luna-Vargas for their assistance in the development of the assays used within this work, and Yiyang Chen for generating recombinant human BAX protein.

AUTHOR CONTRIBUTIONS

Conceptualization, J.N.M. and J.D.G.; Funding Acquisition, J.E.C.; Methodology, J.N.M.; Validation, J.N.M.; Writing, J.N.M. and J.D.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Czabotar, P.E., Westphal, D., Dewson, G., Ma, S., Hockings, C., Fairlie, W.D., Lee, E.F., Yao, S., Robin, A.Y., Smith, B.J., et al. (2013). Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell* 152, 519–531. <https://doi.org/10.1016/j.cell.2012.12.031>.
- Dewson, G., Ma, S., Frederick, P., Hockings, C., Tan, I., Kratina, T., and Kluck, R.M. (2012). Bax dimerizes via a symmetric BH3:groove interface during apoptosis. *Cell Death Differ.* 19, 661–670. Epub 2011 Oct 21. <https://doi.org/10.1038/cdd.2011.138>.
- Garner, T.P., Amgalan, D., Reyna, D.E., Li, S., Kitsis, R.N., and Gavathiotis, E. (2019). Small-molecule allosteric inhibitors of BAX. *Nat. Chem. Biol.* 15, 322–330. Epub 2019 Feb 4. <https://doi.org/10.1038/s41589-018-0223-0>.
- Gavathiotis, E., Suzuki, M., Davis, M.L., Pitter, K., Bird, G.H., Katz, S.G., Tu, H.C., Kim, H., Cheng, E.H., Tjandra, N., and Walensky, L.D. (2008). BAX activation is initiated at a novel interaction site. *Nature* 455, 1076–1081. <https://doi.org/10.1038/nature07396>.
- Gelles, J.D., Mohammed, J.N., Chen, Y., Sebastian, T.M., and Chipuk, J.E. (2022). A kinetic fluorescence polarization ligand assay for monitoring BAX early activation. *Cell Rep. Methods.* <https://doi.org/10.1016/j.crmeth.2022.100174>.
- Hsu, Y.T., and Youle, R.J. (1997). Nonionic detergents induce dimerization among members of the Bcl-2 family. *J. Biol. Chem.* 272, 13829–13834. <https://doi.org/10.1074/jbc.272.21.13829>.
- Pritz, J.R., Wachter, F., Lee, S., Luccarelli, J., Wales, T.E., Cohen, D.T., Coote, P., Heffron, G.J., Engen, J.R., Massefski, W., and Walensky, L.D. (2017). Allosteric sensitization of proapoptotic BAX. *Nat. Chem. Biol.* 13, 961–967. Epub 2017 Jul 10. <https://doi.org/10.1038/nchembio.2433>.
- Sarosiek, K.A., Chi, X., Bachman, J.A., Sims, J.J., Montero, J., Patel, L., Flanagan, A., Andrews, D.W., Sorger, P., and Letai, A. (2013). BID preferentially activates BAK while BIM preferentially activates BAX, affecting chemotherapy response. *Mol. Cell* 51, 751–765. <https://doi.org/10.1016/j.molcel.2013.08.048>.
- Suzuki, M., Youle, R.J., and Tjandra, N. (2000). Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell* 103, 645–654. [https://doi.org/10.1016/s0092-8674\(00\)00167-7](https://doi.org/10.1016/s0092-8674(00)00167-7).
- Tsai, C.J., Liu, S., Hung, C.L., Jhong, S.R., Sung, T.C., and Chiang, Y.W. (2015). BAX-induced apoptosis may be initiated through a conformational selection mechanism. *Structure* 23, 139–148. Epub 2014 Dec 11. <https://doi.org/10.1016/j.str.2014.10.016>.