

Preview

A taste of the early steps in BAX activation with FLAMBE

Melanie Gil,^{1,2,3,5} Marina R. Hanna,^{1,2,3,5} and Vivian Gama^{1,2,3,4,*}¹Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, USA²Neuroscience Graduate Program, Vanderbilt University, Nashville, TN, USA³Vanderbilt Brain Institute, Vanderbilt University, Nashville, TN, USA⁴Vanderbilt Center for Stem Cell Biology, Vanderbilt University, Nashville, TN, USA⁵These authors contributed equally*Correspondence: vivian.gama@vanderbilt.edu<https://doi.org/10.1016/j.crmeth.2022.100190>

The activation of BAX through intricate intramolecular changes is critical for apoptosis. In this issue of *Cell Reports Methods*, Gelles et al. report engineering FLAMBE, an elegant fluorescence polarization ligand assay for monitoring the early activation of monomeric BAX via real-time release of a peptide probe, expanding the repertoire of BAX activation assays to the single-molecule level.

The intrinsic pathway of apoptosis is crucial for embryonic development, tissue homeostasis, and cellular response to irreversible perturbations that converge to the effector protein BAX, a member of the BCL-2 family of proteins. In most cells, BAX is found at the cytosol in an inactive state and must undergo conformational changes to initiate activation and promote its translocation and integration into the outer mitochondrial membrane (OMM) (Chipuk and Green, 2008; Gavathiotis et al., 2008; Hsu and Youle, 1997). In response to intrinsic cellular stressors or developmental signals, BAX is activated by BH3-only direct activator proteins such as BID or BIM at a site located at the N-terminal surface formed by the $\alpha 1$ and $\alpha 6$ sites (Gavathiotis et al., 2008). This initial trigger at the N-terminal surface of BAX is followed by several structural changes including displacement of the $\alpha 1$ - $\alpha 2$ loop, exposure of key BH3 domain residues and the 6A7 epitope, and mobilization of $\alpha 9$ helix from its own hydrophobic binding groove, which promotes translocation and integration into the OMM. These structural changes promote BAX multimerization via $\alpha 6$ and assembly of an oligomeric pore, leading to the release of apoptosis-inducing factors (Dewson et al., 2009). Thus, the ability to detect early BAX activation is essential to capture cellular events that are engaged prior to pore formation and homeostatic responses when BAX undergoes early conformational changes that do not result in mitochondrial membrane permeabilization.

Commonly used approaches to detect BAX activation take advantage of isolated mitochondrial systems and biochemically defined liposomes (Renault et al., 2013). These methods reveal the downstream events that follow BAX activation, such as mitochondrial pore formation and cytochrome *c* release. Conformation-specific antibodies such as the 6A7 and the BH3 antibodies, which detect the exposure of the N-terminal motif (residues 13–19 at the start of $\alpha 1$) and BH3 domain (residues 41–76), respectively, have been useful to detect these early structural changes by immunocytochemistry, flow cytometry, and immunoprecipitation analysis (Chipuk and Green, 2008; Gavathiotis et al., 2008; Hsu and Youle, 1997). There are however some limitations of this approach in high-throughput real-time experiments. Thus, there is a critical need for an assay that examines the intramolecular conformational changes of BAX as it transitions from a cytosolic, inactive monomer to a membrane-associated, pore-forming oligomer. Gelles et al. (2022) introduce a fluorescence polarization ligand assay called FLAMBE for monitoring BAX early activation. Fluorescence polarization is a sensitive and powerful technique used traditionally to study molecular interactions in solution (Moerke, 2009). The principle of this non-radioactive approach centers on a fluorescently labeled small molecule, BAK^{TAMRA}, that when is free in solution, rotates rapidly. Upon excitation by polarized light, the emitted light will be largely depolarized light. When this fluorescent small

molecule is bound to a bigger molecule, such as BAX, the rotational movement of the fluorophore becomes slower due to the significantly reduced rotational speed of the complex. Thus, the emitted light will remain polarized (Figure 1). The binding of a fluorescently labeled small molecule to a protein can be monitored by the change in polarization from low to high. Upon BAX activation, which results in structural rearrangement and multimerization, displacement of the BAK^{TAMRA} probe would be detected as a concomitant decrease in polarization. FLAMBE detects intramolecular structural modifications that monomeric BAX undergoes in response to activators, prior to the large-scale structural changes that trigger its oligomerization followed by permeabilization of the OMM.

As proof of principle, Gelles et al. (2022) interrogate various methods such as the use of detergents, BH3 peptides, and small molecules that have been previously shown to induce BAX activation (Hsu and Youle, 1997; Montero and Letai, 2016; Niu et al., 2017). At low concentrations of the detergent octyl- β -glucoside (OG), active BAX monomers were detected, demonstrating the sensitivity of this tool. Then, using the BIM^{BH3} peptide, a dose-dependent displacement of BAK^{TAMRA} was detected demonstrating the expected functional BAX activation. Even at low concentrations of BIM^{BH3}, an increase in polarization was detected representing the non-triggering BIM binding previously described (Tsai et al., 2015). Experiments exposing BIM^{BH3}-treated BAX to large



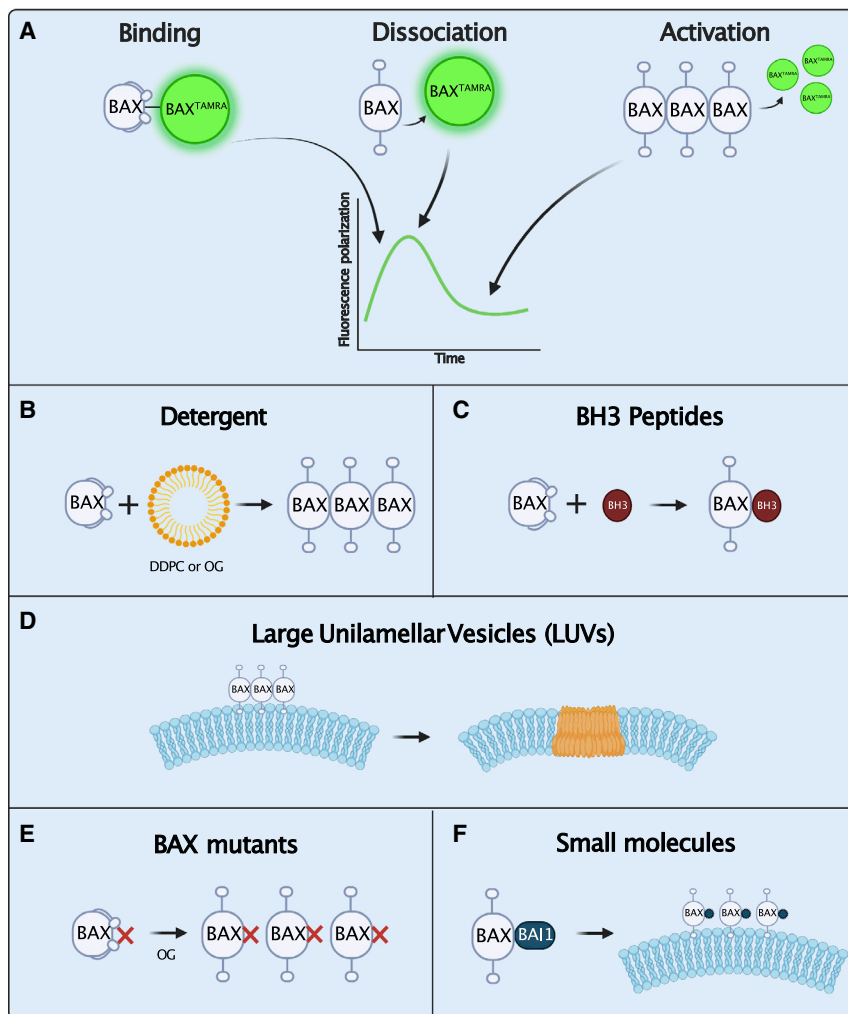


Figure 1. An overview of FLAMBE

(A) FLAMBE is a polarization ligand assay used to examine the early activation of BAX via the release of fluorescently labeled small molecule, BAKTAMRA. Binding of BAKTAMRA to BAX can be monitored by the change in polarization from low to high, allowing for the real-time monitoring of BAX binding, dissociation, and activation of BAX.

(B and C) Activation of BAX was achieved using both (B) detergents and (C) BH3 peptides.

(D) BAX functionalization was tested using large unilamellar vesicles (LUVs).

(E and F) The efficacy of FLAMBE in detecting early activation of BAX was validated via the use of (E) BAX mutants and (F) small molecules.

Created with Biorender (<https://biorender.com/>).

unilamellar vesicles (LUVs) highlighted FLAMBE's capability to distinctively detect the kinetics of triggered (conformationally active) and functionalized (pore-forming) BAX. Insightful experiments with functionally impaired BAX mutants demonstrated the presence of quasi-functional monomers that remained responsive to oligomerization upon treatment with OG, cautioning about the use of detergents to infer structural changes during BAX activation. Additionally, the use of several BAX mutants in the FLAMBE/LUVs exper-

imental paradigm revealed unforeseen structural changes in BAX that can be further investigated in future studies.

Small molecules that alter BAX activity are of significant interest as they may serve as pharmaceutical interventions to modulate apoptosis during tumorigenesis or neurodegeneration. Using FLAMBE, the authors found that the potent BAX inhibitor, BAI1, did not affect triggering or dissociation of BAK^{TAMRA}, suggesting that its potent inhibition activity is due to its interference with later steps of BAX

activation. This plethora of experiments place FLAMBE as a critical tool not only to gain insights into structural changes induced by bona fide BAX interactors but also to probe for the mode of action of candidate small molecules associating with monomeric BAX. Potential applications of the FLAMBE approach could be expanded by incorporating BAX-containing mutations found in tumor cells (Dengler et al., 2021), providing a platform to test novel compounds that could target BAX therapeutically in these cells.

While fluorescent polarization assays as presented by Gelles et al. (2022) are powerful tools to study the kinetic activation of BAX, future iterations of FLAMBE could be optimized to include either cell lysates or membranes to study whether different lipids and/or lipid combinations can engage BAX activation in a BH3-dependent or -independent manner. Ultimately, apart from being central to the commitment to apoptosis in response to cellular stresses, BAX activation is also essential for developmental processes such as epithelial to mesenchymal transitions and inflammation. The described approach will help further understand the inter- and intramolecular mechanisms guiding the highly coordinated BCL-2 family protein-protein interactions and will help set the stage to reveal how apoptotic signaling underlie human development and disease.

ACKNOWLEDGMENTS

Funding was provided by National Institutes of Health (NIH)/National Institute of General Medical Sciences (NIGMS) 1R35GM128915-01 (to V.G.), the NIH/National Institute for Mental Health (NIMH) 1RF1MH123971-01 (to V.G.), the NIH/NICHD Training Program in Stem Cell and Regenerative Developmental Biology 5T32HD007502-24 (to M.H.), and the NIH/NIMH Training in Fundamental Neuroscience 5T32MH064913-19 (to M.G.).

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Chipuk, J.E., and Green, D.R. (2008). How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol.* 18, 157–164. <https://doi.org/10.1016/j.tcb.2008.01.007>.
- Dengler, M.A., Gibson, L., and Adams, J.M. (2021). BAX mitochondrial integration is regulated

- allosterically by its $\alpha 1$ - $\alpha 2$ loop. *Cell Death Differ.* 28, 3270–3281. <https://doi.org/10.1038/s41418-021-00815-x>.
- Dewson, G., Kratina, T., Czabotar, P., Day, C.L., Adams, J.M., and Kluck, R.M. (2009). Bak activation for apoptosis involves oligomerization of dimers via their $\alpha 6$ helices. *Mol. Cell* 36, 696–703. <https://doi.org/10.1016/j.molcel.2009.11.008>.
- Gavathiotis, E., Suzuki, M., Davis, M.L., Pitter, K., Bird, G.H., Katz, S.G., Tu, H.-C., Kim, H., Cheng, E.H.-Y., 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* 2, 100174. <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>.
- Moerke, N.J. (2009). Fluorescence Polarization (FP) Assays for Monitoring Peptide-Protein or Nucleic Acid-Protein Binding. *Curr. Protoc. Chem. Biol.* 1, 1–15. <https://doi.org/10.1002/9780470559277.ch090102>.
- Montero, J., and Letai, A. (2016). Dynamic BH3 profiling-poking cancer cells with a stick. *Mol. Cell. Oncol.* 3, e1040144. <https://doi.org/10.1080/23723556.2015.1040144>.
- Niu, X., Brahmabhatt, H., Mergenthaler, P., Zhang, Z., Sang, J., Daude, M., Ehlert, F.G.R., Diederich, W.E., Wong, E., Zhu, W., et al. (2017). A Small-Molecule Inhibitor of Bax and Bak Oligomerization Prevents Genotoxic Cell Death and Promotes Neuroprotection. *Cell Chem. Biol.* 24, 493–506.e5. <https://doi.org/10.1016/j.chembiol.2017.03.011>.
- Renault, T.T., Floros, K.V., and Chipuk, J.E. (2013). BAK/BAX activation and cytochrome c release assays using isolated mitochondria. *Methods* 67, 146–155. <https://doi.org/10.1016/j.ymeth.2013.03.030>.
- Tsai, C.-J., Liu, S., Hung, C.-L., Jhong, S.-R., Sung, T.-C., and Chiang, Y.-W. (2015). BAX-induced apoptosis can be initiated through a conformational selection mechanism. *Structure* 23, 139–148. <https://doi.org/10.1016/j.str.2014.10.016>.