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BH3 profiling: a functional assay to measure apoptotic priming and dependencies

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Abstract

Apoptosis (programmed cell death) is activated by a wide variety of cellular stresses or insults and is vital for proper mammalian development as well as the maintenance of organismal homeostasis. The apoptosis pathway is also engaged by many common types of anti-cancer therapies and ionizing radiation, which contributes to the regressions of tumors or the toxic side effects of treatment. Due to the importance of maintaining healthy cell survival or the efficient clearance of cancer cells, the BH3 profiling assay was developed to functionally measure the state of the apoptosis pathway in any given cells. This assay involves the exposure of cellular mitochondria, where the BCL-2 family of proteins resides and controls the commitment to apoptosis, to pro-apoptotic BH3 peptides that mimic the activity of endogenous pro-apoptotic proteins. By using either activator or sensitizer peptides, the level of mitochondrial apoptotic priming (proximity to the threshold at which a cell commits to cell death) or dependence on pro-survival BCL-2 family proteins can be determined. Described here are two methods of BH3 profiling that can enable a user to make these functional measurements, which can be useful for predicting cellular responses to pro-apoptotic stressors or therapeutics (BH3 mimetics) that inhibit the activity of pro-survival proteins.

Keywords

mitochondrial apoptotic priming; MOMP; BCL-2 family; chemotherapeutics

1. Introduction

The Apoptotic “Cliff”

Apoptosis is controlled by the BCL-2 family of proteins, which regulate the commitment to programmed cell death [1, 2]. Apoptosis is triggered when the pro-apoptotic, pore-forming proteins BAX and/or BAK are activated by the pro-apoptotic “activators” BIM or BID [3–7] (Figure 1). Upon activation, BAX and BAK homo-oligomerize and cause mitochondrial outer membrane permeabilization (MOMP), which is referred to as the “point of no return” that irreversibly commits a cell to undergoing apoptosis [8, 9]. Once these pores are formed in the mitochondrial outer membrane, cytochrome c is released into the cytosol, resulting in

the formation of the apoptosome, and the subsequent activation of the caspase cascade [8]. At this point, as long as the majority of mitochondria have undergone this process, the cell is committed to apoptotic cell death. The BH3 profiling assay has been designed to measure the amount and type of pro-apoptotic signal that is required for a given cell to trigger apoptotic cell death.

Mitochondrial Apoptotic Priming

Based on our established molecular understanding of apoptosis signaling and a multitude of experimental models, it is clear that the state of this pathway in a cell before it is damaged or stressed can impact cell fate. To illustrate, a living cell that expresses only enough pro-survival proteins to barely buffer endogenous pro-apoptotic signals is considered “primed” for apoptosis (Figure 2). In contrast, a cell that expresses a surplus of pro-survival proteins that can buffer against existing and potentially additional pro-apoptotic molecules is “unprimed.” Finally, cells that do not express sufficient levels of BAX and BAK to undergo MOMP and thus commit to apoptosis are “apoptosis refractory.” Cells that are primed more readily undergo apoptosis in response to damage or stress than unprimed cells [10]. Apoptosis refractory cells are completely protected from pro-apoptotic signaling and unable to die via this pathway without first upregulating BAX and/or BAK [11]. Because the state of the apoptosis pathway can potentially drive cell fate decisions in response to damage or stress, the BH3 profiling assay was developed, which measures apoptotic priming within a given cell.

The BH3 profiling assay is based on measuring the extent of MOMP that occurs in response to pro-apoptotic BH3 peptides (Figure 3), which mimic the activity of full-length pro-apoptotic BH3 only proteins from the BCL-2 family [12]. We have observed the importance of apoptotic priming in determining clinical responses to chemotherapy and ionizing radiation by demonstrating that primed cancers are more sensitive to chemotherapy than unprimed cancers [13–17]. We also found that the level of apoptotic priming within healthy tissues governs their sensitivity to these same treatments, providing a potential explanation for the differential sensitivity of our tissues to damage or stress [11].

Measuring Overall Mitochondrial Apoptotic Priming

The BIM or BID BH3 peptides can be used to determine the overall level of priming in a cell. These peptides are able to bind to any of the anti-apoptotic proteins and can also directly bind and activate BAX and/or BAK. BH3 profiling can also inform us of activation efficiencies: this assay has been previously used to determine that BID preferentially activates BAK while BIM preferentially activates BAX [4]. The PUMA BH3 peptide can also be used to determine the overall priming in a cell as it can bind to any of the anti-apoptotic proteins but cannot activate BAX/BAK directly [12]. While monitoring MOMP, one can deliver titrated doses of the peptides to a permeabilized cell and observe the dose that is required to overwhelm the anti-apoptotic reserves and induce MOMP. The required dose to induce MOMP is inversely correlated with the level of priming of the cell (e.g. high dose of BIM required indicates that the cell has a low level of priming). In addition, by BH3 profiling cells that have been treated with a perturbation (any treatment that induces damage

or stress), one is able to measure changes in apoptotic priming, which may be predictive of eventual cell death [18].

Measuring Anti-Apoptotic Dependencies for Survival

The BH3-only sensitizer proteins can selectively inhibit specific anti-apoptotic proteins but don't activate BAX or BAK as potently as BIM or BID (Figure 3). The peptides that emulate these sensitizer proteins can be used to determine if a cell uses one or multiple specific pro-survival proteins to actively suppress apoptosis. These sensitizers can indirectly induce MOMP as inhibitors of inhibitors. When they bind to the pro-survival proteins, they can release pro-apoptotic molecules including BIM or BID that were being sequestered, allowing for the activation of BAX and/or BAK (Figure 4).

Comparing BH3 Profiling Assays

2. Materials

For both methods of BH3 Profiling, there are 3 main components used: the profiling buffer, the permeabilizing agent digitonin, and peptides or other small molecules used to target BCL-2 family proteins.

2.1. Buffers

1. Mannitol Experimental Buffer (MEB): 10 mM HEPES pH 7.5, 150 mM Mannitol, 50 mM KCl, 0.02 mM EGTA, 0.02 mM EDTA, 0.1% BSA, 5 mM Succinate
2. Newmeyer Buffer: 10 mM HEPES pH 7.7, 300 mM Trehalose, 50 mM KCl, 0.02 mM EGTA, 0.02 mM EDTA, 0.1% BSA, 5 mM Succinate

2.2. Prepared reagents

1. Digitonin: For JC-1 Profiling, prepare 5% (50 mg/ml) solution in DMSO. For iBH3, prepare 1% (10 mg/ml) solution in DMSO.
2. Alamethicin: Dilute to 25 μ M in DMSO.

2.3. Peptides/BH3 Mimetics

Peptide	Sequence	Extinction Coefficient at 280 nm
hBIM	Ac-MRPEIWIAQELRRIGDEFNA-NH ₂	5500 cm ⁻¹ M ⁻¹
hBID-Y	Ac-EDIIRNIARHLAQVGDSMDRY-NH ₂	1490 cm ⁻¹ M ⁻¹
mBAD	Ac-LWAAQRYGRELRRMSDEFEGSFKGL-NH ₂	6990 cm ⁻¹ M ⁻¹
mNoxaA	Ac-AELPPEFAAQLRKIGDKVYC-NH ₂	1490 cm ⁻¹ M ⁻¹
MS-1	Ac-RPEIWMQTQLRRLGDEINAYYAR-NH ₂	8480 cm ⁻¹ M ⁻¹
Puma	Ac-EQWAREIGAQLRRMADDLNA-NH ₂	5500 cm ⁻¹ M ⁻¹
BMF-Y	Ac-HQAEVQIARKLQLIADQFHRY-NH ₂	1490 cm ⁻¹ M ⁻¹
FS-1	Ac-QWVREIAAGLRLAADNVNAQLER-NH ₂	5500 cm ⁻¹ M ⁻¹

Peptide	Sequence	Extinction Coefficient at 280 nm
W-Hrk	Ac-WSSAAQLTAARLKALGDELHQ-NH ₂	5500 cm ⁻¹ M ⁻¹
Puma2A	Ac-EQWAREIGAQARRMAADLNA-NH ₂	5500 cm ⁻¹ M ⁻¹

Ac=Acetyl, NH₂=Amide, -Y and W- designate added residues for UV absorbance measurements at C or N term respectively (see Notes 2).

2.4. Recommended BH3 mimetics used for BH3 Profiling: These drugs should be used at a concentration of no greater than 1 μ M, and can be titrated down from that dose.

ABT-199 (BCL-2 inhibitor)

ABT-263 (BCL-2 and BCL-xl inhibitor)

WEHI-539 (BCL-xl inhibitor)

A-1331852 (BCL-xl inhibitor)

Servier63845 (MCL-1 inhibitor)

JC-1 BH3 Profiling

2.5. Reagent preparation: It is recommended that aliquots of reagents are prepared in advance and stored at -80°C .

1. Oligomycin: Dissolved at 20 mg/mL in DMSO. Adding heat will help accelerate dissolving process.
2. JC-1 dye: Prepare 5 mM in DMSO master stock solution, and store at -80°C . From this master stock, prepare 100 μ M working stocks.
3. B-mercaptoethanol: Prepare 1 M working stocks in water, and store at -20°C .
4. FCCP: Dissolved at 10 mM in DMSO.

2.6. Equipment

1. Black 384 well plate:
Greiner 384 well Black (Fluotrac 200) (Cat#781076)
Corning 384 well Black (Cat#3573)
Fisher Nunc 384 well Black (Cat#12-568-54)
2. Plate reader configured for JC-1 dye fluorescence (Excitation 545 $\pm$ 10 nm, Emission 590 $\pm$ 10 nm) capable of temperature control (32°C).

Flow cytometry-based BH3 Profiling (iBH3 Profiling)

2.7. Buffers

1. FACS Stain Buffer: 2% FBS in PBS
2. Intracellular Stain Buffers: Different intracellular staining buffers can be used with different strength detergents. Select the buffer that is necessary for your

experiment, depending on the requirements of your stains or antibodies. All buffers are 10x final concentration. In order of increasing detergent strength:

3. Standard Intracellular Staining Buffer (10x): Per 50 mL, combine 10 mL FBS, 500 mg saponin, 5 g BSA, and dissolve in 50 mL PBS. Add 100 μ L 10% Sodium Azide. Sterile filter and store at 4°C. Several filters might be necessary. Compare this buffer with BD PermWash (BD Biosciences Cat#554723).
4. Tween20 Intracellular Staining Buffer (10x): Per 50 mL, combine 1 mL Tween20, 5 g BSA, and dissolve in 50 mL PBS. Sterile filter and store at 4°C.
5. Triton-X100 Intracellular Staining Buffer (10x): Per 50 mL, combine 0.5 mL Triton-X100, 5 g BSA, and dissolve in 50 mL PBS. Sterile filter and store at 4°C. After staining with this buffer, the cells must be spun down to remove the Triton-X100 to avoid interfering with the staining intensity of other antibodies.
6. 4% Formaldehyde in PBS
7. Neutralizing (N2) buffer: 1.7 M Tris base, 1.25 M Glycine, pH 9.1. For 50 ml of buffer, add 10.3 g TRIS base (M.W. 121.11) and 4.69 g Glycine (M.W. 75.07). Adjust pH to 9.1, then sterile filter.

2.8. Recommended staining dyes:

1. Zombie viability stain
2. DAPI or Hoechst DNA stain
3. Cell surface markers to identify cells of interest

2.9. Cytochrome c antibody: Recommended antibody is the 6H2.B4 Clone from BioLegend with a fluorescent tag. AlexaFluor 647 and FITC conjugates have been validated in the assay.

2.10. Equipment:

1. Corning Flat bottom 96 well clear NBS
2. Corning Black 384 well NBS
3. Flow cytometer

3. Methods

3.1 JC-1 Profiling (for overview, see Figure 5)

3.1.1 Sample buffer preparation: Calculate required volume of sample buffer. 7.5 μ L of buffer is required per experimental well, and it is recommended to make 20% extra to account for pipetting loss. For every 1 mL of sample buffer, add 4 μ L 5% digitonin, 2 μ L of 20 mg/mL oligomycin, 40 μ L of 100 mM JC-1 dye, 20 μ L 1M β -mercaptoethanol, and 934 μ L MEB/Newmeyer buffer. Set aside and keep out of light.

3.1.2 Prepare treatments and controls: Dilute working peptide stocks/BH3 mimetics to 2x final concentration in MEB/Newmeyer buffer. Add 2 μ L of peptide working stock for every

100 µL of 2x treatment required. 60 µL of each 2x treatment should be prepared for every sample that is being profiled.

3.1.3 Negative controls: DMSO is the carrier for all the treatments and can be used as a charge control. Puma2A is a peptide control that can be used at the highest concentration of any other peptide treatment.

3.1.4 Positive controls: FCCP is a chemical depolarizer that causes rapid loss in mitochondrial trans-membrane potential. Alamethicin is a peptide that forms pores in membranes, causing BAX/BAK-independent MOMP.

3.1.5 Add treatments and controls to plate: Dispense 15 µL of 2x peptides/treatments per well in Black 384 well plate. It is recommended that each treatment is plated in triplicate. For recommended concentrations of peptides, see Notes 1. Tap the plate on all sides to ensure that the treatments fully cover the bottom of the well and are not stuck to the sides of the well.

3.1.6 Collect sample(s): For cell lines, create single cell suspension by repeated pipetting to dissociate clumps. For tissues, homogenize samples by chopping until fine enough to allow for pipette dissociation, or homogenize by dounce homogenizer or similar method. Wash the sample(s) once with PBS before proceeding.

3.1.7 Resuspend sample(s) in MEB/Newmeyer buffer: For most samples, it is recommended that you resuspend to a concentration of no greater than $1-3 \times 10^6$ cells/mL in MEB/Newmeyer. For larger cells, such as many adherent cell lines, 1×10^6 cells/mL should be sufficient, whereas for smaller cells, such as blood cells, you may require up to 3×10^6 cells/mL (see Notes 3).

3.1.8 Prepare sample(s): Add resuspended sample to equal volume of pre-prepared sample buffer. Let mixture rest for 5 minutes to allow for permeabilization of the sample and JC-1 staining.

3.1.9 Plate sample(s): Dispense 15 µL of sample/sample buffer mixture to each well on plate.

3.1.10 Measure fluorescence: Set plate reader temperature to 32°C. Put plate in plate reader, set to record fluorescence every 5 minutes for 180 minutes.

3.1.11 Analyze data: Calculate area under the curve (AUC) values for each treatment. Calculate the effect (Mitochondrial depolarization) of each treatment.

$$\text{Depolarization} = \frac{AUC(\text{Treatment}) - AUC(\text{positive control})}{AUC(\text{negative control}) - AUC(\text{positive control})}$$

The area under the curve of the negative control should be at least 3 times greater than the area under the curve of the positive control. If this is not the case, there might not have been

enough cells loaded or the cells haven't been stained properly. For an example of a JC1 BH3 profile, see Figure 5.

3.2 Flow cytometry-based BH3 Profiling (iBH3 Profiling) (For overview, see Figure 8)

3.2.1 Sample collection: Calculate the number of cells required for profiling. For the 96-well procedure, use 10000-50000 cells per well. For the 384-well procedure, use 5000-25000 cells per well. Wash sample once in PBS. Do not overload the assay plate as further increasing cell number will decrease effective peptide concentration (Figure 9).

3.2.2. Viability staining: Resuspend sample in PBS and proceed with fluorescent viability staining protocol, such as Zombie staining (Biolegend), Live/Dead Staining (Life Technologies), or other viability stains that are retained after fixation. Follow manufacturer protocol.

3.2.3 Cell-surface staining: Resuspend sample in 100 μ L FACS Stain Buffer. Before adding any cell surface staining markers, prepare compensation controls by setting aside 5 μ L of sample for every antibody that will subsequently be used. Add antibodies at pre-determined dilutions. Stain on ice for 30 minutes protected from light. Spin down the samples and rinse once with PBS.

3.2.4 Resuspend cells sample in MEB/Newmeyer buffer

3.3 96-well procedure:

3.3.1 Treatment preparation: Calculate amount of buffer necessary for treatment wells. 25 μ L of buffer is required for every treatment well.

3.3.2 Prepare solution of 0.002% digitonin in MEB/Newmeyer buffer by adding 2 μ L of 1% digitonin for every 1 mL of MEB/Newmeyer buffer.

3.3.3. Add peptide treatments/controls at a 1:50 dilution to the 0.002% digitonin MEB/Newmeyer buffer, leaving the solution at 2x final concentration. For recommended concentrations of peptides, see Notes 1.

3.3.4 Add 25 μ L of cells in MEB/Newmeyer buffer to each well.

3.3.5 Incubate plate for 30-90 minutes at 25°C $\pm$ 3°C. Be aware that peptide responses increase as a function of time and temperature (Figure 8) (see notes 4).

3.3.6 Terminate peptide exposure: Add 15 μ L of 4% formaldehyde in PBS to each well.

3.3.7 Allow plate to rest 10-15 minutes at room temperature.

3.3.8 Neutralize formaldehyde: Add 30 μ L of N2 buffer to each well to end fixation.

3.3.9 Allow plate to rest 10-15 minutes at room temperature.

3.3.10 Prepare intracellular staining solution: For every well, prepare 10 μ L of staining solution.

3.3.11 Add cytochrome c antibody at 1:200-1:400 dilution in 10x Intracellular staining buffer.

3.3.12 Add any other intracellular antibodies at 10x final dilution.

3.3.13 Prepare compensation controls for each antibody.

3.3.14 Add staining solution: Add 10 μ L of intracellular staining solution to each well. Add compensation controls to unstained wells.

3.3.15 Let stain overnight: Cover plate with adherent plate cover and invert plate repeatedly to ensure proper mixing of sample and antibody solutions.

3.3.16 Proceed to Flow Cytometry analysis

3.4 384 well procedure:

3.4.1 Treatment preparation: Calculate amount of buffer necessary for treatment wells. 15 μ L of buffer is required for every treatment well.

3.4.2 Prepare solution of 0.002% digitonin in MEB/Newmeyer buffer by adding 2 μ L of 1% digitonin for every 1 mL of MEB/Newmeyer buffer.

3.4.3 Add peptide treatments/controls at a 1:50 dilution to the 0.002% digitonin MEB/Newmeyer buffer, leaving the solution at 2x final concentration. For recommended concentrations of peptides, see Notes 1.

3.4.4 Add 15 μ L of cells in MEB/Newmeyer buffer to each well.

3.4.5 Incubate plate for 30-90 minutes at 25°C $\pm$ 3°C. Be aware that peptide responses increase as a function of time and temperature (see Notes 4) (Figure 8)

3.4.6 Terminate peptide exposure: Add 10 μ L of 4% formaldehyde in PBS to each well.

3.4.7 Allow plate to rest 10-15 minutes at room temperature.

3.4.8 Neutralize formaldehyde: Add 20 μ L of N2 buffer to each well to end fixation.

3.4.9 Allow plate to rest 10-15 minutes at room temperature.

3.4.10 Prepare intracellular staining solution: For every well, prepare 6 μ L of staining solution. Add Cytochrome c antibody at 1:200-1:400 dilution in 10x Intracellular staining buffer. Add any other intracellular antibodies at 10x final dilution. Prepare compensation controls for each antibody.

3.4.11 Add staining solution: Add 6 μ L of intracellular staining solution to each well. Add compensation controls to unstained wells.

3.4.12 Let stain 1-2 hours: Cover plate with adherent plate cover and invert plate repeatedly to ensure proper mixing of sample and antibody solutions. Overnight staining on a rocker at 4°C recommended.

3.4.13 Proceed to Flow Cytometry analysis

3.5 Flow cytometry analysis:

3.5.1 Identify the cells of interest by excluding particulate matter, doublets, non-viable cells, or any cells that should be excluded based on your specific cell-surface or intracellular staining.

3.5.2 Identify cytochrome c negative population: The positive control wells (alamethicin and unstained FMO) will not have cells that stain positively for cytochrome c. Use these wells to set a gate for cytochrome c negative cells. If there is incomplete cytochrome c loss in the alamethicin well, that indicates that there might too many cells loaded in the well.

3.5.3 Identify cytochrome c positive population: The negative controls wells (DMSO and/or Puma2A) will not cause cytochrome c loss, so all or almost all cells should stain positively for cytochrome c. Use this population to set a gate for cytochrome c positivity. The cytochrome c negative and positive gates are mutually exclusive and should not overlap. A small degree of cytochrome c loss is expected in the negative control wells due to the effects of processing the samples. If there is a high degree of cytochrome c loss in the negative control wells (over 10%), there might be an issue with the cytochrome c staining or overall sample viability.

3.5.4 Run all other treatment wells and look at the amount of cytochrome c negativity caused by the treatments. This can be conveyed in a figure by graphing the amount of cytochrome c negative cells as a percentage of the parental population for each treatment. For a sample gating strategy and iBH3 profile, see Figure 6.

3.5.5 If distinct cytochrome c positive or negative populations are not evident, detect the impact of treatment by looking at the drop in the median fluorescent intensity (MFI) of the cytochrome c fluorophore from the MFI of the negative control.

$$\% \text{Cytochrome c loss} = \frac{MFI(\text{Treatment}) - MFI(\text{positive control})}{MFI(\text{negative control}) - MFI(\text{positive control})}$$

4. Notes

1. Peptide treatment panels may vary based on the type of cell that is being profiled. In order to most accurately and confidently compare the overall priming levels of samples, it is recommended that one obtains the EC50 values for the BIM, BID, and PUMA peptide treatments. Therefore the titrations for these peptides should span from a dose that would induce a complete response to a dose that would cause no response, with doses at full or half-log intervals in between. For highly primed cell types, such as cells of hematopoietic lineage, a lower concentration

of peptides would be required than would unprimed cell types (cells of non-hematopoietic lineage) (Table 2) [11].

2. Peptides should be at least 95% purity, and should be made as TFA salts. Peptides should be diluted in DMSO to a concentration greater than 10 mM to create a master stock. Typically, this can be achieved by diluting the peptide powder at 50 mg/ml in DMSO. The concentration of the peptide should be verified by UV absorption at 280 nm. All master stocks should be stored at -80°C . Working stocks should then be created by diluting the master stock. The working stock should be 100x final concentration and can be stored at -20°C . The working stocks should be approximately 2 weeks worth of peptide, as frequent freeze-thaw cycles should be avoided.
3. Maintaining consistent cell counts across experiments will increase consistency (Figure 9).
4. Temperature and duration of incubation with peptides before fixation can impact the level of cytochrome c release (Figure 10).

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Figure 1: The mitochondrial apoptosis pathway.

In this simplified schematic, cellular stress or damage signals [1] unleash pro-apoptotic proteins (BH3-only "activators" of apoptosis) [2], which can either be bound and sequestered by pro-survival proteins such as BCL-2, BCL-X_L or MCL-1 [3] or activate BAX and/or BAK [4]. Activation of BAX and/or BAK causes mitochondrial outer membrane permeabilization (MOMP), resulting in the release of cytochrome c from mitochondria and consequent activation of caspases [5] for dismantling of the cell.

Figure 2: Potential states of apoptotic priming and competence as determined by BH3 Profiling.

In the BH3 Profiling assay, cells are permeabilized and treated with titrated doses of pro-apoptotic peptides derived from the BH3 domains of BH3-only proteins. Cytochrome c staining is used to monitor mitochondrial permeabilization, which occurs upon activation of BAX or BAK and consequent release of cytochrome c to trigger apoptosis. Mitochondria that have a small reserve of unbound pro-survival proteins are quickly and fully depolarized (loss of cytochrome c) by even low doses of peptides (1-3 μ M) during the 1 hour assay and are thus considered primed for apoptosis. Mitochondria that have a large reserve are unprimed and only respond to large doses of peptides (100 μ M). Mitochondria that lack BAX or BAK are apoptosis incompetent. The pretreatment level of apoptotic priming can determine cell fate in response to pro-apoptotic signaling.

		Activators		Sensitizers					
Protein or BH3 Peptide →		BIM	BID	BAD	N/A	NOXA	MS1 (syn)	HRK	PUMA
Drug (BH3 Mimetic) →				ABT-263	ABT-199	S63845	S63845	WEHI-539	
Pro-Survival	BCL-2								
	BCL-XL								
	BCL-w								
	MCL-1								
	BFL-1								
Pro-Apoptotic, Pore-Forming	BAX								
	BAK								

Strong Inhibition

Weak/No Inhibition

Strong Activation

Moderate Activation

No Direct Activation

Figure 3: Binding pattern for BCL-2 family interactions.

Binding affinities for interactions between BH3 peptides derived from activator or sensitizer BH3-only proteins and their pro-survival and pro-apoptotic partners. (syn) designates a synthetic peptide.

Figure 4: Diagnosing dependencies on pro-survival BCL-2 family proteins.

Using the BH3-only sensitizer peptides, the BH3 profiling assay can detect if a cell has a particular dependence on one or multiple anti-apoptotic BCL-2 family proteins for survival. The sensitizer peptides can selectively inhibit particular anti-apoptotic proteins. If a cell is primed, but does not express a particular anti-apoptotic protein at high levels, the sensitizer peptides will have little effect. If a cell is primed and expresses BCL-2 or MCL-1 at a high level, treatment with the BAD or NOXA peptides respectively will inhibit the anti-apoptotic and allow for the activation of Bax/Bak. Treatment with BAD on an MCL-1 dependent cell will have no effect, as will treatment with NOXA on a BCL-2 dependent cell.

Figure 5: JC1 BH3 profiling workflow.

Cells are collected and prepared into a single-cell suspension. Cells are then permeabilized with digitonin and stained with JC1. Cells are loaded into a 384 well plate that contains BH3 peptides and fluorescence is monitored in a plate reader over 180 minutes.

Figure 6: JC1 BH3 profiles of parental HeLa and BAX/BAK knockout HeLa cells.

Each line on the three-hour time course (left) reflects the fluorescence for a single treatment. The corresponding bar in the bar graph (right) is the difference between the AUC of the negative control and the AUC of the treatment from the three-hour time course. The HeLa parental cell lines are an example of a moderately primed cell line. When BAX and BAK are knocked out from that cell line, the cells become apoptosis refractory, no longer responding to BH3-only peptides BIM and BID.

Figure 7: Flow cytometry BH3 profiling (iBH3) workflow.

Cells are collected and prepared into a single-cell suspension. Cells of interest are then labeled with fluorescent antibodies and washed. Cells are then permeabilized with digitonin and loaded into a 384 well plate that contains BH3 peptides and incubated for 60 minutes. Cells are then fixed and stained for cytochrome c. Cells are analyzed by flow cytometry.

Figure 8: Flow cytometry-based iBH3 profiling analysis.

Single cell analysis enables the user to gate on cellular content, specifically singlets, using forward and side-scatter variables. Specific subtypes of cells can then be identified and gated on for further analysis. After selecting the population of interest, the differences in cytochrome c negative cells can be examined between different treatments and populations. In this example, only DAPI positive cells (permeabilized cells with full DNA content) are included in the cytochrome c panel. The negative control treatment (DMSO) population is mostly cytochrome c positive. Increasing dosage of the BIM-BH3 peptide results in increased cytochrome c negativity. Alamethicin causes total cytochrome c loss.

Figure 9:
Effective peptide concentration decreases as cell concentration increases.

Figure 10:
Increased incubation temperature, time increases cytochrome c release.

Table 1:

Comparison of BH3 profiling protocols and their application.

Assay	JC-1 Plate-based BH3 Profiling (JC-1 BH3 Profiling)	Flow Cytometry BH3 Profiling (flow BH3 Profiling)
Application	Homogenous samples	Heterogeneous samples (can probe subsets of cells within samples)
MOMP Readout	Mitochondrial trans-membrane potential BAX/BAK activation will cause pore formation, decreasing trans-membrane potential.	Cytochrome c release BAX/BAK activation will cause pore formation, causing cytochrome c release from mitochondria. These cells will therefore no longer have cytochrome c and will not stain positively for cytochrome c.
Advantages	<ul style="list-style-type: none"> • Less processing necessary • Time course provides more information about timing of response to peptides • Higher throughput 	<ul style="list-style-type: none"> • Use viability stains/DNA content stains to ensure cell viability • Identify specific cell types within sample by FSC, SSC, cell surface markers, and by intracellular staining
Disadvantages	<ul style="list-style-type: none"> • Heterogeneity within sample will not be evident or accounted for 	<ul style="list-style-type: none"> • One time point (end-point measurement) • Lower throughput

Table 2:

Recommended peptide treatment panels for different cell types.

		Bim	Bid	Puma	Bad	Hrk	Noxa	MS-1	BMF	FS-1
Primed cell peptide panel	Maximum concentration (μM)	10	10	100	100	100	100	10	100	10
	Minimum concentration (μM)	0.01	0.01	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Unprimed cell peptide panel	Maximum concentration (μM)	100	100	100	100	100	100	30	100	30
	Minimum concentration (μM)	0.1	0.1	10	10	10	10	1.0	1.0	1.0