METHOD 2: iBH3 PROFILING

Unlike JC-1 plate based BH3 profiles, the iBH3, or intracellular BH3, method relies on the measurement of retained cytochrome c rather than potential. Because it is a flow cytometry assay, cell surface marking as well as some intracellular stains are compatible with this method making it suitable for co-cultures and mixtures of cells found in primary tissues samples. Because it terminates in fixation, it can be used with infectious samples including those bearing pathogens such as HIV.

iBH3 Common Materials

MEB Buffer
4% Formaldehyde in PBS
Treatment Stocks peptide or compounds at 100X final concentration (typically in DMSO)
5% Digitonin 50 mg/mL digitonin in DMSO
'N2' Buffer [1.7M Tris, 1.25M Glycine pH 9.1]
10X CytoC Stain Buffer (10% BSA, 2% Tween20, PBS, filtered)
Anti-Cytochrome C antibody Clone 6H2.B4 labeled with a fluorescent tag

Cell Surface Staining Materials (optional)
2% FBS in HBSS (Or your preferred cell surface staining buffer)
Live/Dead stains
Cell surface markers (i.e. CD3, EpCAM, etc…)

Order of Events
1. Cell surface staining / Viability marking
2. Peptide Exposure
3. Fixation
4. Fixative Neutralization
5. Intracellular staining
6. FACS analysis

Notes regarding scale and concentration limits

The procedures listed here have been tested in tube, 96 well, and 384 well formats. With overnight staining for cytochrome c and a dilution of 1:400, the assay can tolerate cell densities from 0.5-4 million cells per mL. Exceeding the upper limit will cause a reduction in the dynamic range as measured by the median fluorescence intensity of the cytochrome c staining of positive and negative controls.

Controls

A matched isotype control for the cytochrome c antibody can be used as a negative control in an iBH3 profile. The MOPC clone has been tested by the Letai lab but does occasionally stain brighter than some treatments resulting in cytochrome c release values greater than 100%. This can be remedied by using a fluorescence minus one (FMO) control in which the cytochrome c antibody is omitted without the addition of an isotype.

Alternatively, 15-25 μM Alamethicin can serve as a positive control for cytochrome C release. Alamethicin can be handled in the same manner as the peptides. Alamethicin will produce a staining pattern more similar to full release due to peptides like BIM, and it is highly recommended as the only means to discriminate failure to release cytochrome c and operator error in situations where BAX and BAK are absent or non-functional.

1% DMSO or the inert PUMA2A peptide at the highest used peptide concentration are typically used as controls for complete cytochrome c retention. MEB alone can also be used.

Fixation

Typically completed by adding formaldehyde to a final concentration of 1-2% for 10 minutes. Formaldehyde is typically added as a 4% or 8% solution in PBS. Fixation as long as 30 minutes before N2 addition has not had significant negative impacts on performance.

Viability and Cell Surface Staining

1. Viability stains (ZombieAqua (BioLegend), Live/Dead (Life Technologies), ect.. are reactive dyes that are retained after fixation. These should be used before antibody staining to avoid chemically altering the antibodies. Follow manufacturer instructions, but in general this is completed by:
    a. Suspending the cells in an amine-free buffer (PBS or HBSS) without serum or BSA.
    b. Reactive dye is added on ice or at room temperature for 15-30 minutes
    c. The cells are spun down and can be washed or suspended in buffer with BSA or FBS which will quench the reaction

2. Antibody staining is typically done on ice in 1%BSA or 2% FBS in HBSS for 30 minutes protected from light. Antibody titer should be determined before profiling. Standard compensation controls are those typically used as in standard FACS experiments
    a. Suspends cells in 1%BSA or 2%FBS in HBSS with additional blocking agents if needed (i.e. FcR Block)
    b. Add antibodies and stain for 30 min on ice
    c. Spin down cells and wash once with HBSS

3. Proceed to the iBH3 variant that suits the sample size and desired throughput

Tube-Based procedure

1. Add 100 uL of 0.002% (w/v) digitonin in MEB with treatments diluted to 2X their final concentration in each FACS tube

2. Suspend cells in MEB buffer (with or without prior staining), 100 uL for each tube. Making an extra sample worth of cell suspension will reduce the chance of running out of cells while dispensing them due to pipette error or dead volumes. Suspend cells at 2X final desired density.

3. Add 100 uL of cells in MEB to each tube and flick or gently vortex to mix

4. Wait 30-90 minutes at 25°C +/- 3 °C

a. Increased temperature will accelerate cytochrome c release. Near 30°C or above, spontaneous release may occur.
b. 30 minutes works well for highly sensitive or fragile cells.
c. 90 minutes is best for studying slow responding or highly unprimed cells
d. Most samples will fall into the 45-60 minute window.

5. Add 67 uL **of 4% Formaldehyde** in PBS to each tube to fix the cells and terminate the peptide exposure

6. Wait 10 minutes at room temperature

7. Add 67 uL of **N2 buffer** to each tube to neutralize the formaldehyde and terminate fixation

8. Wait at least 5 minutes

9. Prepare 40 uL per tube of **10X CytoC Stain Buffer** with a 1:40 dilution of the anti-Cytochrome c antibody

10. Add either 40 uL of a 1:40 dilution of an isotype control in 10X CytoC Stain Buffer to the negative control tube to make an isotype control OR add 40 uL of the stain buffer with no antibodies added to serve as an FMO control

11. Add 40 uL of the diluted anti-cytochrome c antibody to the remaining tubes

12. Mix gently and let stain at least 1-2 hours at room temp. Overnight staining at 4°C is highly recommended.

13. Proceed to FACS Analysis

Multiwell / High-Throughput Procedures

96 Well Procedure

1. Add 50 uL of 2X treatment in 0.002% digitonin in MEB to each well

2. Add 50 uL of cells (stained or not) at 2X final concentration in MEB to each well

3. Wait 30-90 minutes at 25°C +/- 3 °C
    a. Increased temperature will accelerate cytochrome c release. Near 30°C or above, spontaneous release may occur.
    b. 30 minutes works well for highly sensitive or fragile cells.
    c. 90 minutes is best for studying slow responding or highly unprimed cells
    d. Most samples will fall into the 45-60 minute window.

4. Add 33 uL **of 4% Formaldehyde** in PBS to each tube to fix the cells and terminate the peptide exposure

5. Wait 10 min at room temperature

6. Add 33 uL of **N2 buffer** to each tube to neutralize the formaldehyde and terminate fixation

7. Wait at least 5 minutes

8. Prepare 20 uL per tube of **10X CytoC Stain Buffer** with a 1:40 dilution of the anti-Cytochrome c antibody

9. Add either 20 uL of a 1:40 dilution of an isotype control in 10X CytoC Stain Buffer to the negative control wells to make an isotype control OR add 40 uL of the stain buffer with no antibodies added to serve as an FMO control

10. Add 20 uL of the diluted anti-cytochrome c antibody to the remaining wells.

11. Cover and let stain at least 1-2 hours at room temp. Overnight staining at 4^{o}C is highly recommended.
    a. The use of an adhesive sealing film is recommended to prevent evaporation or spills / contamination during cytochrome c antibody incubation but is not necessary to the procedure

12. Proceed to FACS Analysis

384 Well Procedure

Special Note: Because the wells of a 384 plate are tall and narrow, density gradients can form particularly when N2 or intracellular stain buffer is applied. After the plate is sealed, it must be mixed to ensure even mixing of all components.

1. Add 15 uL of 2X treatment in 0.002% digitonin in MEB to each well

2. Add 15 uL of cells (stained or not) at 2X final concentration in MEB to each well

3. Wait 30-90 minutes at 25^{o}C +/- 3 ^{o}C
    a. Increased temperature will accelerate cytochrome c release. Near 30^{o}C or above, spontaneous release may occur.
    b. 30 minutes works well for highly sensitive or fragile cells.
    c. 90 minutes is best for studying slow responding or highly unprimed cells
    d. Most samples will fall into the 45-60 minute window.

4. Add 10 uL **of 4% Formaldehyde** in PBS to each tube to fix the cells and terminate the peptide exposure

5. Wait 10 min at room temperature

6. Add 10 uL of **N2 buffer** to each tube to neutralize the formaldehyde and terminate fixation

7. Wait at least 5 minutes

8. Prepare 10 uL per tube of **10X CytoC Stain Buffer** with a 1:40 dilution of the anti-Cytochrome c antibody

9. Add either 10 uL of a 1:40 dilution of an isotype control in 10X CytoC Stain Buffer to the negative control wells to make an isotype control OR add 40 uL of the stain buffer with no antibodies added to serve as an FMO control

10. Add 10 uL of the diluted anti-cytochrome c antibody to the remaining wells.

11. Cover and let stain at least 1-2 hours at room temp. Overnight staining at 4°C is highly recommended.
    a. The use of an adhesive sealing film is recommended to prevent evaporation or spills / contamination during cytochrome c antibody incubation but is not necessary to the procedure
    b. Mixing in small wells can be incomplete. After the sealing film is firmly in place, it is advisable to mix the plate by inverting 4-5 times before overnight staining.

12. Proceed to FACS Analysis

FACS Analysis for iBH3

There are two main ways to quantify cytochrome c release in the iBH3 assay: normalized median fluorescence intensity (MFI) or by setting gates. Normalization of the MFI values has the same requirements as the calculations used in normalizing plate-based data in that a positive and negative control value are required. DMSO or PUMA2A controls can serve as a maximum MFI control while a fluorescence minus one (FMO) in which the cytochrome c antibody is omitted can be used as the lower bound. Nothing will ever reach the FMO MFI and so no value higher than 100% can occur. However, alamethicin at 25 μM can also be used, and it will look more like a high-dose BIM treatment.

Pos Ctrl = Positive release controls. FMO or alamethicin
Neg Ctrl = Negative release controls. DMSO or PUMA2A

$$\%\, Cytoc\ \ rome\ c\ loss = 1 \quad \frac{MFI_{Sample} \quad MFI_{Pos\ Ctrl}}{MFI_{Neg\ Ctrl} \quad MFI_{Pos\ Ctrl}}$$

It is important to note that slight changes in curve shape can shift the MFI. For instance, it has been observed that PUMA2A controls generally have a narrower curve than DMSO. While the reason for this is not currently understood, the effect is that a PUMA2A control will often have a lightly higher MFI value in the cytochrome c channel and thus serves as a better control for zero release.

An alternative to using the MFI is to draw a gate in the cytrochrome c channel. While it is possible to do this in one dimension using a simple histogram, histograms can sometimes be deceptive because they cannot account for the size of the cell and the resulting change in background staining and mitochondrial content that a two dimensional gate can provide. Staining with control antibodies such as isotypes or MnSOD revealed a strong correlation with side scatter (SSC) and staining. This is a common occurrence in many staining situations, and thus SSC can serve as a suitable second dimension for cytochrome c release.

Two gates can be made that generally complement each other. These are cytochrome c positive or cytochrome c negative gates, and these gates can be defined from the PUMA2A or alamethicin controls respectively. In either case, a gate is created from a SSC vs Cytochrome c plot. This can be done from a dot plot, but a density plot is often helpful in isolating the real shape of the populations. Cells will generally show a strong positive correction between SSC and cytochrome c staining, and this a diagonal gate will be made along the boundary of the control population. In each case, cell health and gating leading into this plot may result in some outliers. In general, if 95% of the control population fits in the respective gate, the gate can be considered suitable.

In the event that many cells in the PUMA2A control are falling into the region of alamethicin, it may be necessary to use a viability marker to remove dead or dying cells from the gating. As a general rule, however, cells should be as healthy as possible to ensure an accurate measure of priming. This is not always possible with primary samples, and so viability staining with agents like DAPI or fixable dyes like Biolgend's Zombie series can help remove most of these cells. If the input may have more than 30-40% dead cells, a dead cell removal column system such as the one offered by Miltenyi is a good option. This make a highly necrotic sample that would ruin surface staining become a much more manageable sample. I have used this kit to clean up samples from xenografts in the past, and this negative selection kit made it possible to accurately stain surface markers as well as get a clean BH3 profile from an otherwise unusable sample.

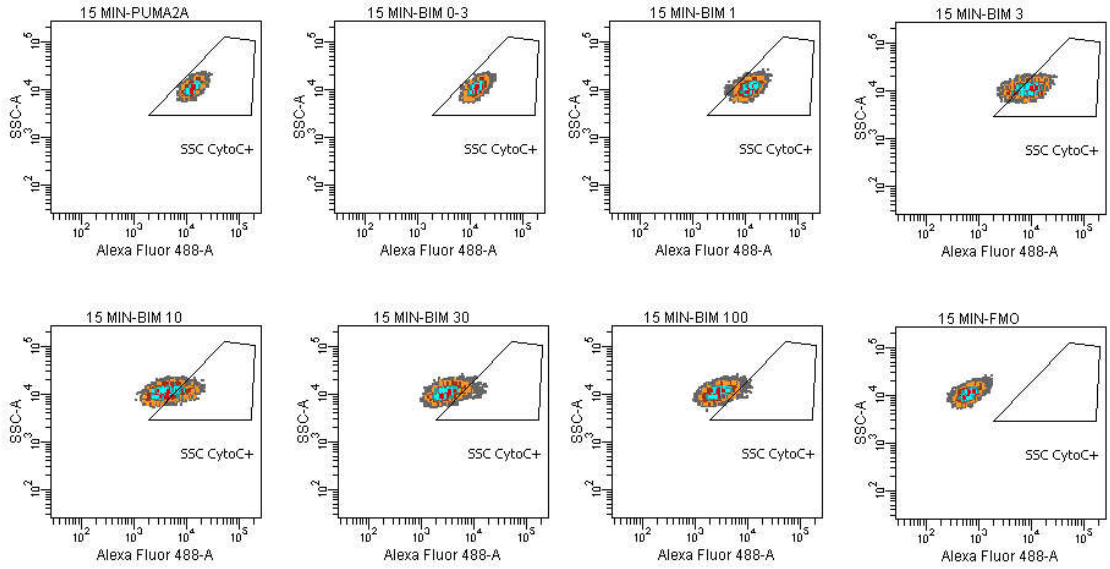

Figure 5: Using gates to quantify cytochrome c release. From upper left moving right across the rows are plots of SSC on the Y-axis vs cytochrome c on the X-axis. The PUMA2A control on the upper left shows how to cut the diagonal gate to select cytochrome c positive cells. Note that increasing BIM concentration causes more cytochrome c release with each dose escalation. Finally, note how far to the left FMO appears relative to BIM 100. This is normal. If alamethicin is used as a control, it will appear more like BIM 100.

It is very important to remember that an iBH3 profile measures the amount cytochrome c retained in the mitochondria. The pores made by digitonin in the plasma membrane are so large that any cytochrome c released from the mitochondria into the cytoplasmic space will immediately leave the cell completely.