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Flow Cytometry-Based Detection and Analysis of BCL-2 Family Proteins and Mitochondrial Outer Membrane Permeabilization (MOMP)

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Abstract

The BCL-2 family of proteins orchestrates a complex signaling network that governs the balance between cellular survival and death. A comprehensive understanding of the mechanistic interactions between these proteins continues to evolve in normal and malignant cells. The functional variation by individual BCL-2 proteins in different cell types has driven clinical therapeutic development in targeting individual BCL-2 members with the goal of fine-tuning cell death in diseased cells. Given the importance of understanding and validating the effect of activating or inhibiting BCL-2 protein interactions in individual cells, the methods used to measure apoptotic cell death have undergone increased scrutiny. Here, we describe two in vitro flow cytometry-based methods that are useful in measuring BCL-2 proteins and mitochondrial-based cell death in complex cell populations.

Keywords

BCL-2 proteins; Intracellular staining; Flow cytometry; Mitochondria outer membrane permeabilization (MOMP); Apoptosis

1 Introduction

Since ancient times, there has been great interest in the study of cell death. While Hippocrates first used the term “apoptosis” in the fourth century B.C., it was not until hundreds of years later that a more complete understanding of the cell death pathway was gleaned and appropriate tools to study it were developed. In the 1970s, apoptosis was formally described as a unique variation of cell death distinct from necrosis [1,2]. Apoptosis is a form of altruistic cell suicide and is a crucial homeostatic process essential for the clearance of superfluous cells, especially in the context of development, immunity, and malignancy [3]. Although molecular hallmarks of apoptosis were known for many years, only over the past 30 years have the proteins governing apoptosis been characterized. How these proteins regulate cell death in normal and diseased cells continues to be an active area of research.

Apoptosis can be initiated by two related but nonredundant pathways that ultimately coalesce at the level of the mitochondrion and result in the activation of caspases that mediate cell destruction [4]. The *extrinsic* pathway is mediated through cell membrane receptors (FAS or other members of the TNFR family) where ligand binding causes adaptor

protein association and caspase-8-activation [5]. The *intrinsic* pathway operates in response to various intracellular stressors like cytoskeletal damage, ER stress, and growth factor withdrawal, and is mediated by the BCL-2 family of proteins [6]. The first identified member of this family, BCL-2, was found near the anomalous junction of chromosomes 14 and 18 (t(14;18)) in follicular lymphoma [7, 8]. Unlike other previously identified oncogenes that act by increasing cellular proliferation, overexpression of BCL-2 was found to block cell death and hence promote cell survival [9]. Rapid discovery of other BCL-2 family proteins ensued and continues to expand. There are now at least sixteen members of the BCL-2 family that have been identified and characterized to regulate apoptosis at some level [10].

The BCL-2 family of proteins can be divided into three functional categories, each containing up to four BCL-2 homology (BH) domains (Fig. 1). Despite sharing amino acid similarity, these homologs have both proapoptotic and antiapoptotic functions and provide their own series of checks and balances on one another to exquisitely regulate cell death. Multidomain proapoptotic effector proteins (BAK and BAX) coalesce at the mitochondria when activated and form a pore that leads to mitochondrial outer membrane permeabilization (MOMP) [11]. Multidomain antiapoptotic proteins (BCL-2, BCL-X_l, BCL-W, BLF-1, MCL-1) sequester BAX and BAK, preventing their activation and subsequent MOMP. The interactions between the proapoptotic and antiapoptotic multidomain proteins are regulated by proteins containing only the BH3 domain (BH3-only proteins; BIM, BID, PUMA, BAD, BIK, BMF, HRK, NOXA) [12]. BH3-only proteins are further divided into “direct activators” or “sensitizers” based on their ability to target antiapoptotics as well as directly bind and activate BAK and BAX or only bind the antiapoptotic proteins respectively (Fig. 2). MOMP is considered a “point-of-no-return” for cell viability and can be measured once completed through classical apoptotic morphological and cellular changes like cell shrinkage, plasma membrane blebbing, cellular detachment, nuclear condensation, nuclear DNA fragmentation, and externalization of phosphatidylserine (PS) on the outer cellular membrane [13]. However, the real-time measurement of apoptosis is more difficult given variations in apoptotic resistance, the speed of cell death following MOMP, and complexity of the interactions between BCL-2 proteins within individual cells.

Many different techniques have been used to study cell death with varying specificity to apoptotic and nonapoptotic mechanisms [14]. Techniques range from light, electron, and fluorescence microscopy for identification of cellular morphological changes and fluorescently labeled cell death-associated proteins (e.g. PS exposure and annexin V positivity), to immunoblotting for identification of mitochondrial-released or caspase-cleaved proteins in cell extracts, to a wide-range of commercially available kits intended to measure aspects of cell death like loss of membrane integrity, caspase activity, and ATP/ADP ratio [15]. Given the complexity of cell death, a single “end point” assay is unable to accurately and specifically differentiate apoptosis from autophagy or necrosis. This is of particular importance, especially with the emphasis on BCL-2 mimetic drug discovery and validation [4,16,17]. Here we discuss two assays able to measure intracellular BCL-2 family protein content and mitochondrial-based cell death using flow cytometry. These techniques have become important validation tools in examining activation of the intrinsic apoptotic pathway.

1.1 Intracellular Flow Cytometry for BCL-2 Proteins

The real time activation of cell death can occur quickly or slowly. Understanding the quantitative BCL-2 family protein abundance as a function of time and treatment are valuable tools to comprehensively evaluate the mechanism(s) responsible for apoptotic triggering. Intracellular flow cytometry confers many advantages on the study of apoptosis. There are many commercially available kits that enable detection of caspase activity, and techniques like Western blotting that can probe for different members of the BCL-2 family of proteins, cleavage of caspases, or release of cytochrome *c* from the mitochondria to the cytoplasm. However, intracellular flow cytometry allows for rapid, multiparametric analysis of cells at multiple time points. Flow cytometry also allows the isolation of specific cell populations based on their cellular membrane protein expression profile and can be used to detect small populations of cells that would otherwise be missed using other techniques.

1.2 Mitochondrial Membrane Depolarization

A proton gradient exists across the inner mitochondrial membrane as a result of proton pumping by the respiratory chain apparatus embedded within the membrane [18, 19]. Conformational changes and homooligomerization of activated BAK and BAX lead to opening of the outer mitochondrial membrane through the formation of a pore [20, 21]. Once formed, the BAK/BAX pore permeabilizes the mitochondrial outer membrane leading to release of proteins normally sequestered within the intermembrane space, such as cytochrome *c*. Leakage of protons depolarizes the mitochondrion and can be visualized using flow cytometry. This can help differentiate apoptosis from necrosis [22, 23]. Detection of MOMP relies on the use of cationic dyes, such as rhodamine 123, tetramethylrosamine ethyl ester (TMRE), 5,5′,6,6-′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), or diOC carbocyanine family probes that congregate within the negatively charged mitochondrial matrix [24–26]. An intact membrane results in dye accumulation and a positive fluorescent signal. If the outer membrane loses its integrity and negative charge, the dye will no longer associate with the mitochondria, resulting in a loss of fluorescence intensity. Many dyes start with a positive signal in a single fluorescent channel and lose that signal, however there are dyes that undergo a shift from one emission to another (JC-1 is an example of this) [27]. Therefore it is important to consider the expected emission of each dye and how it will overlap with any additional fluorophores being used in the experiment. Pros and cons of many dyes used to assess mitochondrial membrane potential can be found in a thorough review by Cottet-Rousselle et al. [25].

2 Materials

Prepare all solutions using analytical grade reagents. Use and store all reagents at 4 °C/ice, unless otherwise indicated.

2.1. Intracellular Flow for BCL-2 Proteins

1. FACS buffer: PBS, 1% FBS, (Optional: 0.1% sodium azide) (*see* Note 1).

2. Cellular permeabilization kit/buffers. We use the FOXP3 Fix/Perm Kit (ThermoFisher, formerly eBioScience): 10× Perm Buffer, Fixation/Permeabilization Concentrate, Diluent.

3. BCL-2 antibodies (*see* Note 2):

   Murine and Human BCL-2 proteins commonly detected:

   (a) BCL-2:

   - PE Hamster Anti-Mouse Bcl-2 Set (3F11; comes with separate isotype control) BD 556537.
   - PE Mouse Anti-Human Bcl-2 Set (Bcl-2/100; comes with separate isotype control) BD 556535.

   (b) BCL-X_L:

   - (54H6) Rabbit mAb (PE conjugate) CST 13835S (detects human and murine isoforms).

   (c) MCL-1:

   - (Y37) Rabbit mAb (Alexa Fluor 647 conjugate) Abcam ab197035 (detects human and murine isoforms).

   (d) BIM:

   - (C34C5) Rabbit mAb (PE conjugate) CST 12186S (detects human and murine isoforms).

   (e) BAX:

   - (6A7) mAb (requires a secondary antibody conjugated to a fluorophore) ThermoFisher MA5–14003 (detects human and murine activated BAX isoforms).

   (f) BAK:

   - (Ab-1) mAb (requires a secondary antibody conjugated to a fluorophore) Millipore TC-100 (detects human activated BAK isoform).
   - (NT) mAb (requires a secondary antibody conjugated to a fluorophore) Millipore ABC12 (detects human and murine activated BAK isoforms).

4. IgG control antibodies:

   (a) Rabbit (DA1E) mAb IgG Isotype Control Alexa(R)647 CST 2985S.

   (b) Rabbit (DA1E) mAb IgG XP(R) Isotype Control (PE Conjugate) CST 5742S.

   (c) PE Hamster Anti-Mouse BCL-2 Isotype Control (in kit listed above).

5. Optional: Secondary antibodies (if primary BCL-2 antibodies are used that are not conjugated to fluorophores) (*see* Note 3).
6. Optional: Viability Marker such as LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (ThermoFisher L10120). We routinely use this to gate out late apoptotic/necrotic cells.
7. 1.2 mL FACS tubes.
8. Flow cytometer (LSRII or similar).

2.2 Mitochondrial Outer Membrane Permeabilization (MOMP)

1. Cationic dyes such as Rhodamine 123, tetramethylrosamine ethyl ester (TMRE), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide (JC-1), or DiOC family dyes can be used for this protocol. Rhodamine 123 at a working solution of450 nM will be used as detailed below (*see* Note 4).
2. FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydra-zone), 1 mM stock.
3. DMSO or other suitable negative control treatment.
4. Optional: Viability marker such as LIVE/DEAD Fixable Far Red Dead Cell Stain Kit (ThermoFisher L10120). We routinely use this to gate out late apoptotic/necrotic cells.
5. 1.2 mL FACS tubes.
6. Flow cytometer (LSRII or similar).

3 Methods

Carry out all procedures at room temperature but keep reagents cold on ice, unless otherwise specified. All washes should be performed by spinning the cells down at 300–400 × *g* for 5 min and flicking the FACS tubes to dispose of the supernatant.

This protocol is optimized for cells in suspension and not for adherent cells. If adherent cells must be used, trypsinization should be avoided or at least minimized if possible to prevent anoikis-induced cell death [28, 29]. Although trypsinization usually does not affect traditional cell death assays (e.g., MTT, XTT assays), it can affect proteins bound to the cellular membrane and change the permeability profile of cells.

3.1 Intracellular Flow Cytometry for BCL-2 Proteins

1. Count cells and aliquot up to 1×10^6 cells per treatment into FACS tubes. Save two aliquots of cells for a fixed and unfixed unstained control (*see* Note 5).
2. Spin down the cells at 300–400 × *g* for 5 min and flick the tubes to dispose of the supernatant. Wash cells once with 200 μL PBS.
3. Optional: Stain cells for extracellular antibodies (*see* Note 6). After incubation, add 200 μL PBS and spin down, then wash once with 200 μL PBS.

4. Optional: Add Live/Dead stain. Check the manufacturer's protocol to ensure that the Live/Dead stain is compatible with permeabilization, such as the LIVE/DEAD Fixable Far Red Stain Kit. For this kit, dilute the Live/Dead stain 1:10,000 in PBS. Add 100 μL to the cells and incubate for 15 min on ice. After incubating, add 400 μL of PBS and spin down and flick to remove the supernatant. Wash cells once with 200 μL PBS and proceed to the fixation step.

5. Prepare the Fixation/Permeabilization working solution: one part concentrate to three parts diluent (*see* Note 7).

6. Add 100 μL of Fixation/Permeabilization solution to the cells. Incubate for 60 min on ice. Keep cells in the dark if they have been stained for extracellular antigens or live/dead.

7. Dilute the 10× permeabilization buffer with Millipore water. After incubation, add 200 μL of permeabilization buffer to each sample and spin down. Wash once with 200 μL permeabilization buffer.

8. Prepare antibody solutions for the BCL-2 proteins of interest. In this protocol, antibody solutions are prepared specifically for detection of BCL-X_L, MCL-1, BIM, and BCL-2, however, there are other BCL-2 proteins that have validated antibodies for use in flow cytometry (listed in the methods). A typical dilution is 1:50 in permeabilization buffer, but an antibody titration may need to be performed for best results. Follow manufacturer's protocol closely as some antibody solutions may already be optimized and should not be diluted. For example, the anti-murine BCL-2 antibody listed above comes in a kit that has been optimized and prediluted along with its own isotype control. For this antibody, add 20 μL of the antibody solution straightaway to each sample. Additionally, an IgG isotype control should be prepared for each protein as well, using the same dilutions as the primary antibodies.

9. Add 50 μL of antibody to each sample (with the exception of the BCL-2 antibody and its control, see previous step). Incubate with intracellular antibodies for 60 min in the dark on ice. After incubation, add 200 μL permeabilization buffer and spin down (*see* Note 8).

10. Wash once with 200 μL permeabilization buffer. Resuspend cells in 200 μL FACS buffer.

11. Analyze via flow cytometry. Start with the unstained controls and adjust voltages so that there is no positive signal from the cells. The cells should be in a roughly uniform population between 0 and 10^2 in the unstained samples. If staining cells with multiple colors at a time, run single stain or compensation bead controls and adjust compensation accordingly.

12. Once the voltage and compensation has been set, run the samples. Vortex each sample before loading it onto the sample injection port. Run the samples on low if possible. Try to be consistent with the number of events collected for each sample; a value of 10,000 events per sample is typically sufficient.

13. Data analysis can be performed on FlowJo, FACSDiva, or a similar flow analysis software. Start with compensation if looking at multiple colors in one sample. For FlowJo, a compensation matrix can typically be generated automatically through the software. Start with the IgG controls and gate out the following: cell type of interest >single cells >live cells (if applicable) (Fig. 3). Once the population of interest has been gated, switch the flow plot to a histogram with the fluorophore of interest on the x-axis. For positive cells, draw a gate that only includes ~1% of the isotype control (Fig. 4). Click and drag the entire gating scheme to the rest of the samples. Repeat this gating strategy for all proteins/ fluorophores. (Fig. 4) (*see* Note 9).

3.2 Mitochondrial Membrane Depolarization

1. Following treatment with an apoptotic stimulus as indicated, count cells and resuspend in FACS tubes to a concentration of 1×10^6 cells/mL. Wash cells once with 200 μl PBS.
2. Aliquot two tubes of cells for a negative (no treatment; polarized membrane) and positive (total mitochondrial membrane depolarization) control.
3. Optional: Add Live/Dead stain. If permeabilizing the cells, check the manufacturer's protocol to ensure that the Live/ Dead stain is compatible with permeabilization, such as the LIVE/DEAD Fixable Far Red Stain Kit. If using this kit, dilute the Live/Dead stain 1:10,000 in PBS. Add 100 μL to the cells and incubate for 15 min on ice. After incubating, add 400 μL of PBS and spin down and flick to remove the supernatant. Wash cells once with 200 μL PBS and proceed to the next step.
4. Add 100 μL of PBS to each sample. For the negative control, treat with DMSO or another suitable control solution. Treat cells with 2 μL of 1 mM FCCP as a positive control. FCCP is a respiratory uncoupler that quickly depolarizes the mitochondrial membrane. Depolarization should be complete by the time the cells are done staining (*see* Note 10).
5. Stain the cells with 25 μL of450 nM rhodamine 123 for 30 min at room temperature in the dark. Do not wash prior to flow cytometric analysis.
6. Analyze via flow cytometry. Start with the unstained controls and adjust voltages so that there is no positive signal from the cells. The cells should be in a roughly uniform population between 0 and 10^2 in the unstained samples. If staining cells with multiple colors at a time, run single stain or compensation bead controls and adjust compensation accordingly.
7. Once the voltage and compensation has been set, run the samples. Vortex each sample before loading it onto the sample injection port. Run the samples on low if possible. Try to be consistent with the number ofevents collected for each sample; a value of 10,000 events per sample is typically sufficient.
8. Data analysis can be performed on FlowJo, FACSDiva, or a similar flow analysis software. Start with compensation when looking at multiple colors in one

sample. For FlowJo, a compensation matrix can typically be generated automatically through the software. Start with the positive and negative control samples. The DMSO treated sample (negative control) can be used to generate a "Polarized" gate and the FCCP treated sample (positive control) can be used to generate a "Depolarized" gate. Remaining samples should either overlap with one of these two samples or fall somewhere in between if partial membrane depolarization occurred (Fig. 5).

4 Notes

1. Sodium azide is useful to help preserve antibody–antigen interactions; however, it also affects cell viability. We have success in performing these assays in FACS buffer without sodium azide, and therefore it is listed as an optional addition.
2. These antibodies are to be used in conjunction with other fluorophores if the need to use additional colors arises. If already using the PE and Alexa Fluor 647 channels for other antibodies, different fluorophores can be used, or unconjugated antibodies can be used with a number of secondary antibody-conjugated fluorophores. These may need to be titrated and optimized for best results. When choosing anti-bodies for flow panels, be aware of the available lasers on the cytometer being used, and be sure that the fluorophores have an emission frequency that will be detected by the cytometer.
3. For primary antibodies conjugated to fluorophores, use IgG controls conjugated to the same fluorophore. If using unconjugated primary antibodies followed by incubation with a secondary antibody conjugated to a fluorophore, set aside a sample as a "no primary" control. Incubate this sample with only the secondary antibody.
4. Other dyes can be substituted for rhodamine 123, such as JC-1, TMRE, and DiOC family dyes. Concentration of the dyes may need to be adjusted for best results.
5. If there is a small amount of cells being tested, the unstained control samples do not have to be at the same density as the other samples. These samples will be used to adjust the voltages of the cytometer and ensure the fixation and permeabilization steps were effective.
6. If there are samples with multiple cell types of interest and there are plans to stain for cell surface markers, stain extracellular antigens as a separate step prior to fixation. A typical stain time would be 30 min on ice. Once the staining process has begun, the cells should be kept in the dark whenever possible.
7. Fixation and permeabilization will cause the cells to become smaller, therefore expect a shift to the left on the forward/side scatter plot during analysis.
8. This protocol was performed using antibodies already conjugated to fluorophores. If an unconjugated antibody is used an additional incubation step will be required for the conjugated secondary antibody.

9. Validation of results using Western blot analysis is a good way to confirm that the intracellular flow is working properly. If using limited number of cells, antibody validation can be performed on other cell types (e.g., immortalized cell lines). There are many cell lines in which the BCL-2 protein levels have been described. These can be useful tools when checking antibody optimization.

10. If there is overlap between the DMSO and FCCP treated controls, and the FCCP-treated sample is still emitting a strong positive signal, the mitochondrial membrane may not be fully depolarized. A longer incubation with the FCCP prior to staining or a higher concentration of FCCP can be used for larger peak separation.

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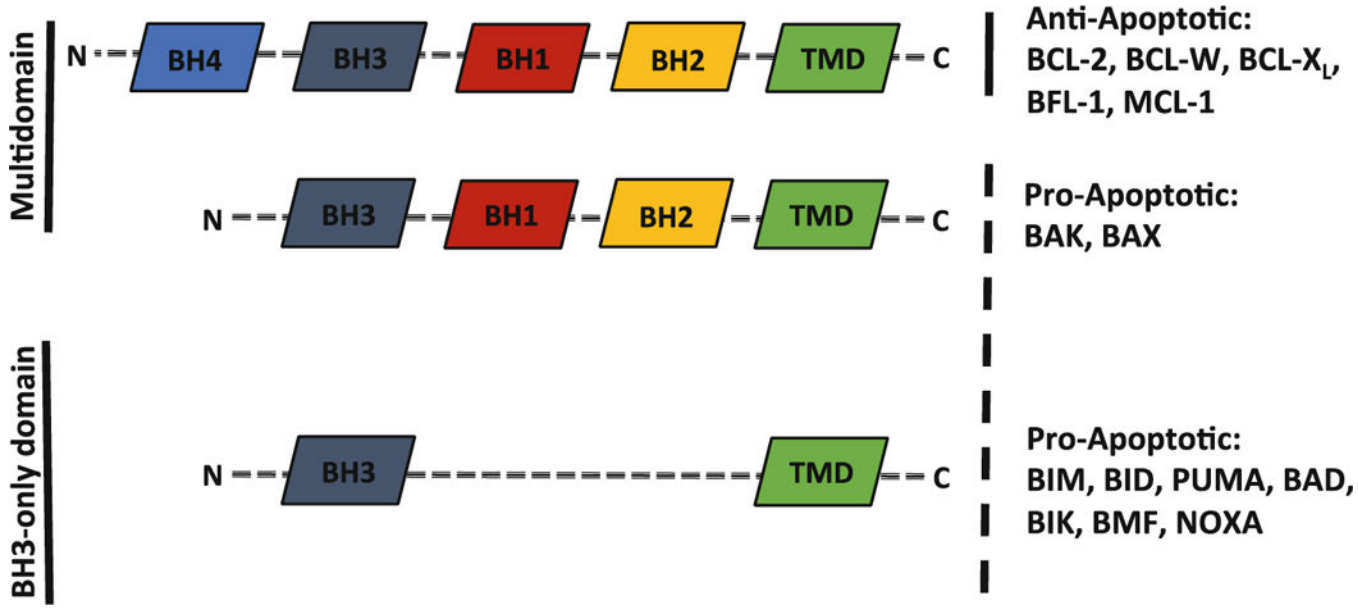

Fig. 1.

BCL-2 family members are characterized by the BH motifs they contain (labeled) and functions they perform. BH3-only BCL-2 proteins (e.g., BAD, BID, BIM) generally have proapoptotic functions as either activators or sensitizers while multidomain BCL-2 proteins can be either anti-apoptotic (e.g., BCL-2, BCL-W, BCL-X_L) or proapoptotic (e.g., BAK, BAX). Members of each domain and functional group differ in size (indicated by dashed lines)

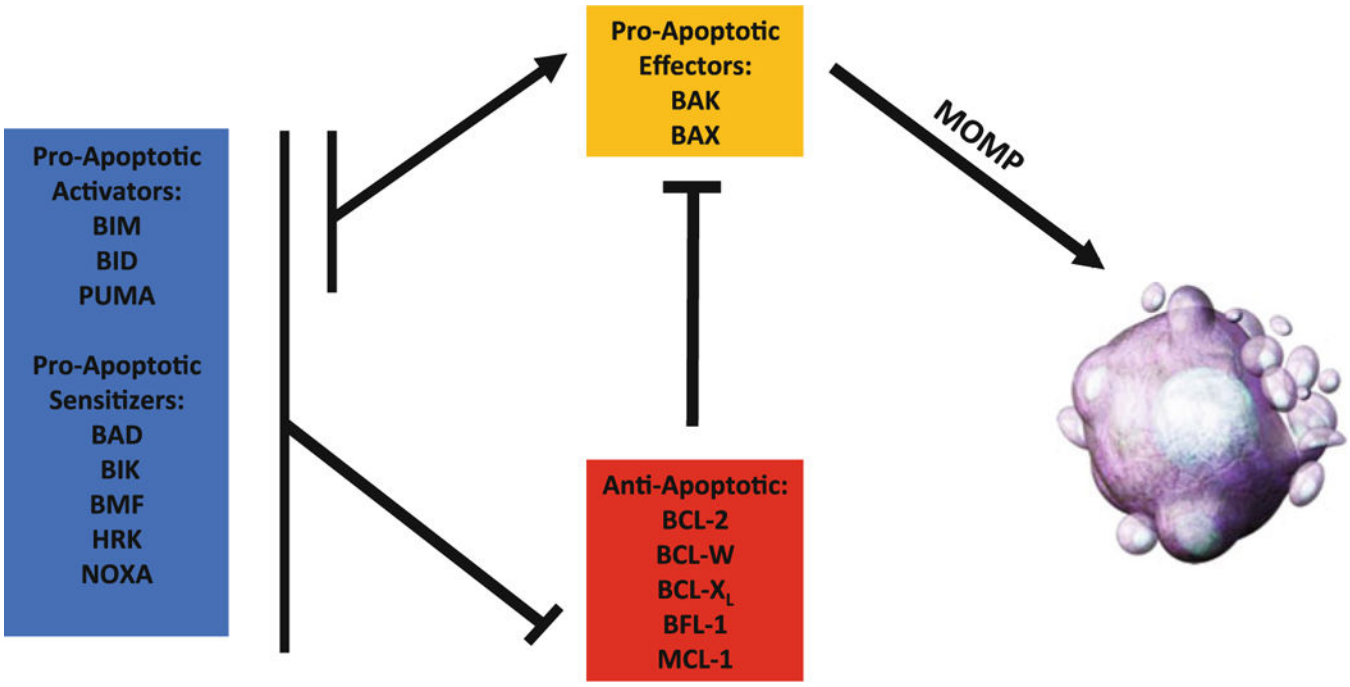

Fig. 2.

BH3-only proteins can act either as sensitizers by inhibiting the anti-apoptotic multidomain proteins (e.g., BCL-2, BCL-W, BCL-X_L) or as activators by directly activating proapoptotic multidomain proteins (BAK and BAX). Ultimately, BAK/BAX activation and pore formation leads to mitochondrial outer membrane permeabi-lization (MOMP), cytochrome *c* release, activation of the caspase network, and cell death. BCL-2 family member functional roles and relationships are indicated as either activating (arrow) or inhibitory (crossed)

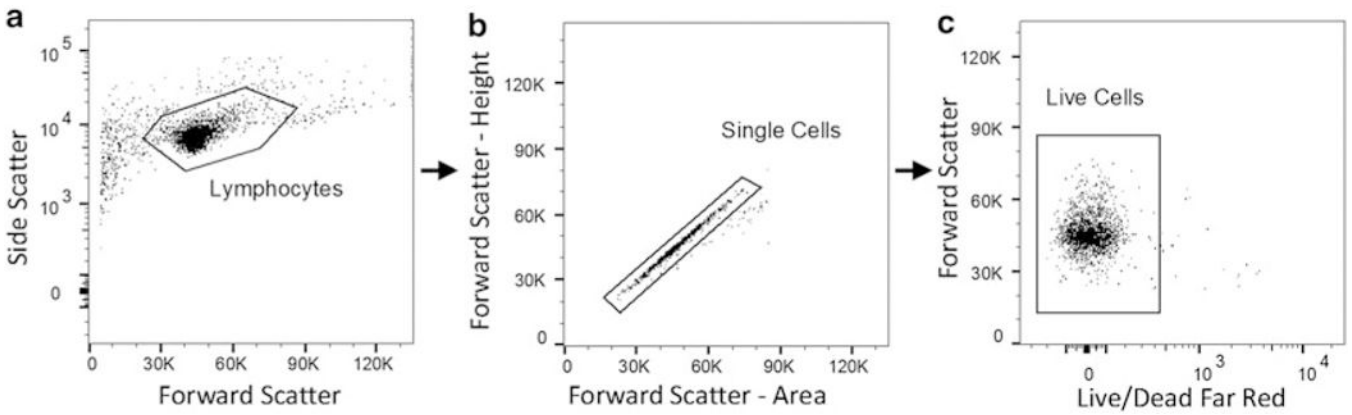

Fig. 3.

Example gating strategy using FlowJo software. (**a**) Start with forward scatter by side scatter and draw a gate around the cell type of interest, such as lymphocytes shown here. (**b**) Next analyze "forward scatter – area" by "forward scatter – height" to exclude any cell doublets. Make a gate around events that fall along the line $y = x$; these are single cells. If a live/dead stain has been used, draw a final gate around live cells. (**c**) In this example, dead cells fluoresce in the far red (APC-Cy7) channel, and therefore a gate has been drawn around the live (APC-Cy7^{Neg}) cells

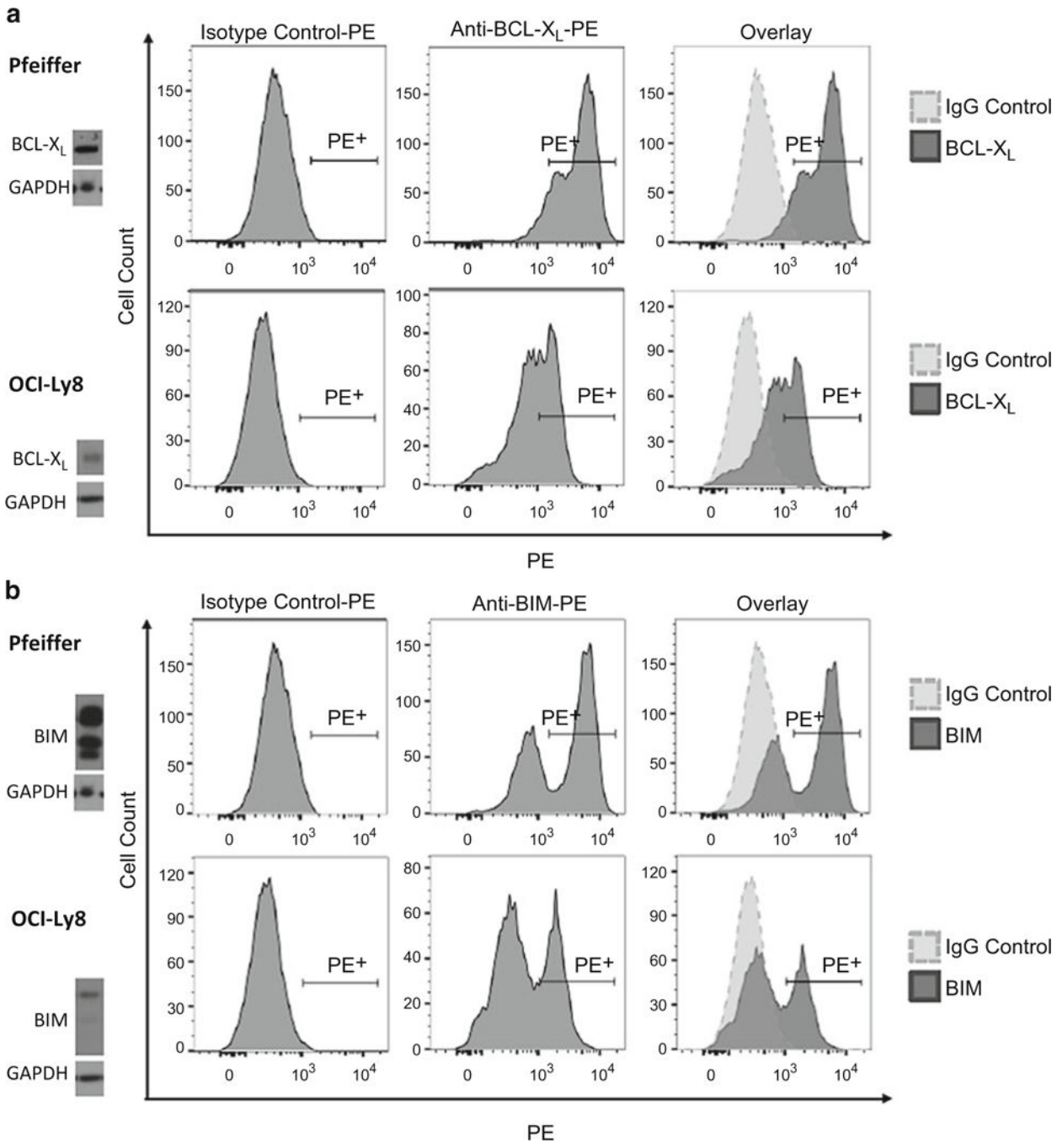

Fig. 4.

Representative examples of intracellular flow for (**a**) BCL-X_L and (**b**) BIM in human diffuse large B cell lymphoma cell lines Pfeiffer and OCI-Ly8. The positive gate is drawn using the IgG isotype control (left column). This gate is copied over to the samples incubated with either BCL-X_L-PE or BIM-PE antibodies (center column). An overlay of the samples with their isotype controls is shown in the right column. Western blotting of each cell line reveals the relative protein levels of BCL-X_L and BIM. As reflected in protein lysate and

intracellular flow, Pfeiffer has higher relative amounts of BCL-X_L and BIM compared to OCI-Ly8

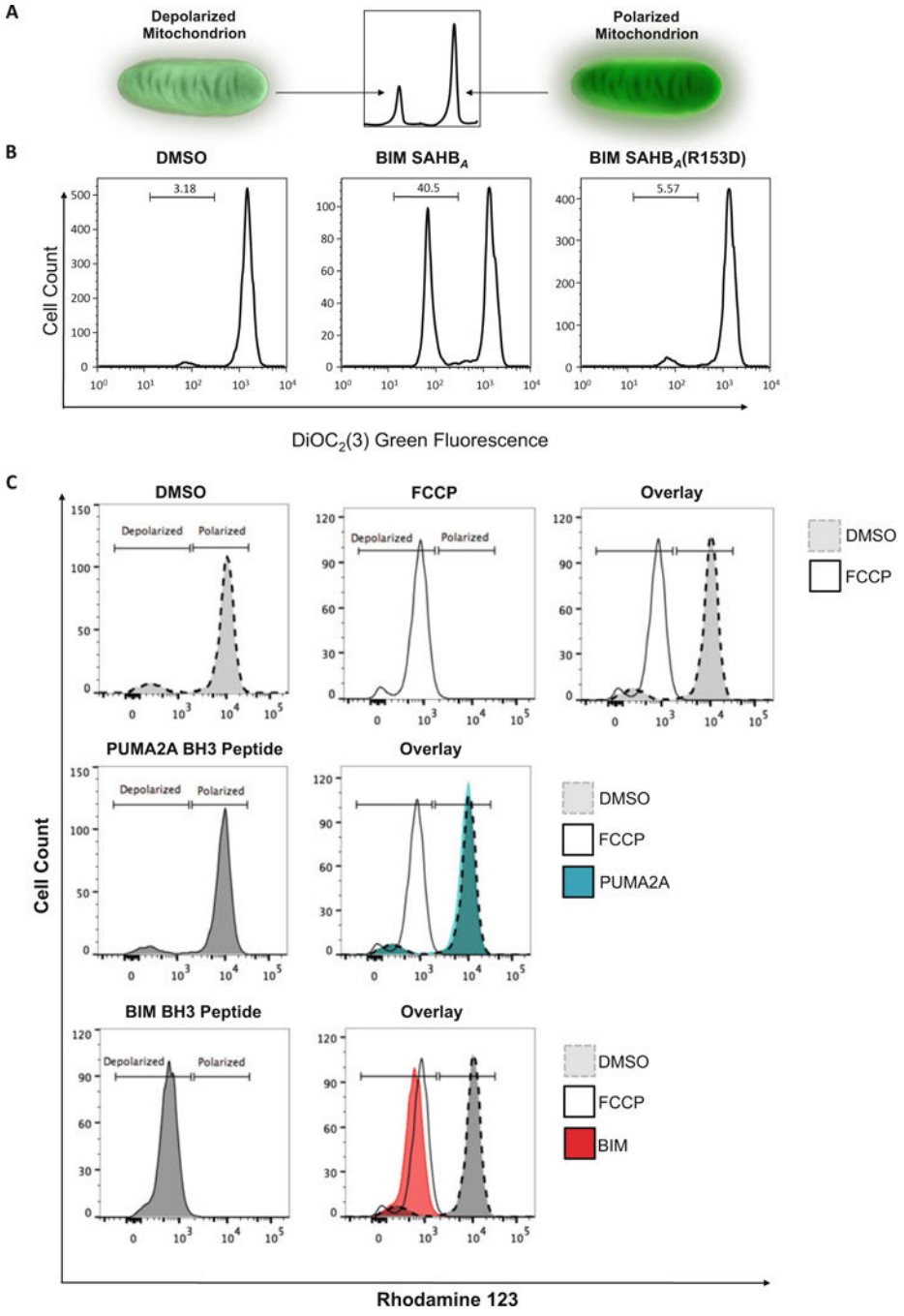

Fig. 5.

Measurement of mitochondrial outer membrane permeabilization (MOMP). (**a**) The general principle of how cationic dyes are used to measure MOMP is shown. As the membrane depolarizes and loses its negative charge, the cationic dyes will no longer associate with the mitochondria and the fluorescent signal will be lost. (**b** and **c**) Two examples of MOMP measurement are shown. (**b**) In the first example, MOMP is measured after treating cells with a stabilized alpha helix of BCL-2 domain (SAHB) modeled after the BH3-only protein BIM, which is known to induce apoptosis [30–33]. Treatment with BIM SAHB$_A$ (middle)

causes partial membrane depolarization compared to DMSO (left) while treatment with a point mutant control, BIM SAHB$_A$(R153D), results in no depolarization. (**c**) The second example demonstrates the use of BH3 peptides optimized to test the dependency of different cell types on specific anti-apoptotic proteins, a technique known as BH3 profiling [27, 34]. DMSO and FCCP are used as negative and positive controls respectively. These controls are overlaid and used as a reference guide in the measurement of the level of depolarization from other BH3 peptide treatments. The PUMA2A peptide serves as a negative control and does not cause membrane depolarization. BIM BH3 uniformly leads to complete depolarization in all cell types, and therefore overlaps with the FCCP treated sample