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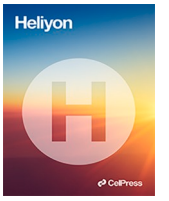

Research article

Flow cytometry-based quantitative analysis of cellular protein expression in apoptosis subpopulations: A protocol

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ABSTRACT

Flow cytometry techniques utilizing dual staining with annexin V and propidium iodide (PI) provide a robust method for quantitatively analyzing apoptosis induction. Annexin V binds phosphatidylserine exposed on the outer leaflet of the plasma membrane during early apoptosis, while PI permeates late apoptotic/necrotic cells. Simultaneous staining allows differentiation of viable, early apoptotic, and late apoptotic/necrotic populations. This approach can be enhanced by using fluorochrome-conjugated antibodies to stain specific proteins, enabling the simultaneous tracking of protein expression changes in defined cell subpopulations during apoptosis. This multiparametric approach provides key insights into signaling regulation and the mechanisms underlying the apoptotic response to cytotoxic treatments. Here we present a protocol that combines annexin V-FITC/PI staining with APC-conjugated antibody labeling in MDA-MB-231 breast cancer cells treated with doxorubicin. This protocol enables both the quantitative assessment of apoptosis induction and the tracking of decreased CD44 expression from viable to apoptotic cells. This protocol also provides guidelines for appropriate filter selection, compensation controls, gating strategies, and troubleshooting. This robust protocol holds significant potential for elucidating signaling networks involved in apoptosis and therapeutic resistance across various cellular models.

1. Introduction

Apoptosis is a highly coordinated and regulated process of programmed cell death that is critical for maintaining tissue homeostasis and proper developmental processes. Dysregulation of apoptotic signaling pathways is implicated in various pathological conditions such as cancer, where resistance to apoptosis is a hallmark of malignancy [1]. Therefore, understanding the dynamics of apoptosis induction and signaling on a cellular level provides key insights into disease mechanisms and therapeutic responses [2]. Flow cytometry techniques utilizing dual staining of fluorescently-labeled annexin V and propidium iodide (PI) have emerged as indispensable tools for quantitatively analyzing apoptosis [3–5]. Annexin V preferentially binds to phosphatidylserine exposed on the outer

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leaflet of the plasma membrane during early stages of apoptosis. Conversely, PI is a DNA intercalating agent that is restricted from viable cells with intact membranes but permeates late apoptotic and necrotic cells [6]. Simultaneous staining with these fluorophores enables distinct identification and quantification of viable, early apoptotic, and late apoptotic/necrotic cell populations [7]. Furthermore, this technique can be further enhanced by integrating staining for specific cellular proteins using fluorochrome-conjugated antibodies, allowing apoptosis induction and protein expression to be evaluated in defined cell subpopulations.

The MDA-MB-231 cell line was selected as a model for this protocol because it is a widely used triple negative breast cancer line that has been well-characterized to possess high CD44 and low CD24 expression, a phenotype associated with breast cancer stem cells (CSCs) [8,9]. Compared to other commonly used breast cancer lines like MCF7 and T47D, the MDA-MB-231 line exhibits a more aggressive phenotype, higher invasive potential, and an abundance of CSC markers [10]. Since CSCs play a major role in cancer progression, therapeutic resistance, and metastasis, studying signaling pathways and apoptosis in the MDA-MB-231 CSC-enriched model provides insights that are broadly relevant to understanding and overcoming these processes in aggressive breast cancers [11]. Cell lines originating from various breast cancer subtypes may display variations in their precise cancer stem cell (CSC) marker profiles. However, the methodologies outlined in this protocol are designed to be flexible by allowing the selection of suitable cell line models and the optimization of antibody staining panels. It is crucial to emphasize that while the chosen markers are pertinent to the specified cell line derived from a particular breast cancer type, the stemness markers could vary across different breast cancer subtypes and other types of cancers.

This Protocol provides a detailed methodology for the dual staining cell lines (e.g. MDA MB-231) using annexin V conjugated with fluorescein isothiocyanate (FITC) and PI, along with allophycocyanin (APC)-conjugated antibodies that target markers such as CD44 and CD24. We present guidelines for appropriate filter selection based on the excitation and emission profiles of each fluorophore. This robust experimental approach generates quantitative multiparametric data on the dynamics of apoptotic cell death and concurrent alterations in protein expression induced by cytotoxic treatments. This protocol can be broadly adapted to characterize signaling regulation across various cancer models, providing valuable biological insights with implications for identifying therapeutic targets and improving anti-cancer strategies.

2. Materials and reagents

The cell culture materials used in this protocol included the MDA-MB-231 breast cancer cell line cultured in complete Dulbecco's Modified Eagle Medium containing high glucose, 10 % fetal bovine serum, and 1 % penicillin/streptomycin. Apoptosis was induced using agents such as doxorubicin. Cells were manipulated using standard cell culture materials including phosphate buffered saline without calcium and magnesium, 5 mL serological pipettes, pipette tips, T25 flasks, a refrigerated benchtop centrifuge, and a vortex mixer. Staining procedures utilized phosphate buffered saline with 25 mM calcium chloride, 0.25 % Trypsin/EDTA, fluorescein isothiocyanate-conjugated Annexin V, propidium iodide, allophycocyanin-conjugated antibodies targeting CD44 and CD24 markers, and bovine serum albumin. Flow cytometry was performed using flow tubes, 40 μm cell strainers, a cytometer equipped with 488 nm blue and 633 nm red lasers, and analysis software such as FlowJo.

2.1. Cell culture and drug treatment

1.1 Culture cell lines of interest (i.e. MDA-MB-231) in a recommended complete medium (i.e. DMEM with 10 % FBS) at 37 °C with 5 % CO_2.
1.2 Seed cells (1×10^6) in T25 flasks to achieve 70–80 % confluence after overnight incubation.
1.3 Treat cells with apoptosis-inducing agent (i.e doxorubicin) by adding directly to complete medium at optimized concentrations (i.e. IC50, 1 μM)) and incubating for determined time periods (e.g. 48 h).
1.4 Include control wells without drug treatment (seed the control cells at lower density (1×10^5) to avoid cell death due to over-confluency condition).

2.2. Cell harvesting and staining

2.1 Collect the old media from the flask in 15 mL tubes (to collect the dead cells), using surgical pipette.
2.2 Wash both control and treated cell samples with PBS (calcium- and magnesium-free) two times, then harvest by trypsinization with 0.05 % trypsin/EDTA for 3 min (or until the cells detached) and neutralize with 1 mL of complete medium.
2.3 Transfer cells to the 15 mL labeled tubes from step 2.1, and pellet by centrifugation at 300×*g* for 5 min at room temperature.
2.4 Aspirate supernatant carefully without disturbing the cell pellet.
2.5 Wash cells by re-suspending in 2 mL PBS (with 25 mM $CaCl_2$). Move 1 mL from the control into new tube as label it as unstained cells (this is the first control tube for Flow cytometry machine). Take 3 × 0.5 mL from the treated tube (the most extreme case) into three new tubes, and label them as PI single stain, FITC single stain, and APC single stain (will serve as compensation control tubes for the Flow cytometry machine). Top up the new tube with PBS to be at same volume with the other tubes, for balancing the centrifuge)
2.6 Centrifuging the tubes at 300×*g* for 5 min, and then discard the supernatant.

2.7 Prepare the Annexin staining solution, by adding 5 μl of Annexin V-FITC conjugate to 1 mL of binding buffer (PBS with 25 mM $CaCl_2$), re-suspend cell pellets in 100 μL Annexin-staining solution. Incubate at room temperature in the dark for 15 min (Note: do not add to PI single stained, APC single stained, and unstained tubes).
2.8 Prepare the PI staining solution by adding 5 μl of PI to 1 mL of PBS to be in a concentration of (1 μg/mL), re-suspend cell pellets in 100 μL PI- staining solution. Incubate at room temperature in the dark for 15 min (Note: do not add to Annexin V-FITC single stained, APC single stained, and unstained tubes).
2.9 Wash cells by adding 2 mL PBS, centrifuging at 300×*g* for 5 min and then discard the supernatant.
2.10 Fix the cells by re-suspending the cell pellets with 80 % methanol for 10 min.
2.11 Subject tubes to Centrifugation at 300×*g* for 5 min ad discard the supernatant.
2.12 If the protein of interest is localized within the cytoplasm, then permeabilization with 1 % PBS-Trion X-100 for 15 min is needed.
2.13 Re-suspend cell pellets in PBS +5 % BSA +0.3 M glycine as blocking agent, to reduce the unspecific binding. Incubate at room temperature in the dark for 30 min.
2.14 Re-suspend cell pellets in PBS +5 % BSA containing optimal concentrations of APC-conjugated antibodies (e.g. anti-CD44, anti-CD24). Incubate at room temperature in the dark for 30 min.
2.15 Wash cells with 2 mL PBS to remove unbound antibody. Centrifuge at 300×*g* for 5 min and discard supernatant.
2.16 Re-suspend cells in 300 μL PBS +1 % BSA for analysis.
2.17 Filter cell suspensions through 40 μm strainers prior to flow cytometry.

2.3. Flow cytometry analysis

3.1 Use the single-stained controls to perform compensation and generate a compensation matrix correcting for spectral overlap between detection channels.
3.2 Analyze experimental samples, acquiring 10,000 events per sample. Gate cell populations based on the healthy cells:
- Viable cells (Healthy): Annexin V-FITC negative, PI negative
- Early apoptotic cells: Annexin V-FITC positive, PI negative
- Late apoptotic cells: Annexin V-FITC positive, PI positive
- Necrotic cells: Annexin V-FITC negative, PI positive

3.3 Apply previously generated compensation matrix to all samples.
3.4 Identify and isolate the background signal of APC fluorescence using the histogram gated population from the unstained tube.
3.5 Evaluate specific protein-APC fluorescence within each histogram gated population to assess expression profiles.

2.4. Data interpretation

4.1 Quantify percentages of viable, early apoptotic, late apoptotic, and necrotic cells in each sample.
4.2 Compare protein-APC expression levels by measuring the median fluorescence intensity (MFI) between different cell populations in control vs treated groups (in case the histogram data is even or normally distributed).

It is important to notice that in a symmetrical (normal) distribution, the median, arithmetic mean, mode, and geometric mean are all very similar, even though, the International Clinical Cytometry Society, the experts recommend using the median (preferred) or the geometric mean (second best choice) for the evaluation of MFI on a logarithmic. However, if we look at a skewed distribution, the mean will be pulled towards the skewed area. To compensate for this, the geometric mean (gMFI) is often used to account for the log-normal behavior of flow cytometry data. The MFI should not be used in a bimodal distribution, because any average only applies to normal distributions, and a bimodal population is not normal by definition. Instead, gating each population and presenting percentages will provide much more useful information. Furthermore, normalizing the median FI to the number of events is not necessary because the median is already a robust statistic that is less influenced by skew or outliers. Therefore, normalizing the median FI to the number of events would not provide additional benefits.

2.5. Statistical analysis

To assess the statistical significance of differences in apoptosis subpopulations and protein expression among the samples, we performed a two-way ANOVA using GraphPad Prism version 9.5, followed by multiple comparisons (using Tukey's test. A significant level of $P < 0.05$ was applied. All experiments were performed with 3 independent biological replicates per group, and 2 technical repeats were conducted per biological replicate. The data is presented as the mean ± standard deviation (SD).

2.6. Troubleshooting

The process of staining cells with PI, Annexin V-FITC, and an APC-conjugated antibody involves multiple steps, and various issues can arise during the process. Below are some possible problems that could emerge throughout this procedure, along with troubleshooting suggestions:

1. **High Background or Nonspecific Staining**:

   Troubleshooting:

   - Ensure thorough washing steps between each staining and blocking step to remove unbound reagents.
   - Optimize blocking agent concentration and duration to minimize nonspecific binding.
   - Include appropriate negative controls to identify background staining sources.
   - Check the specificity of the APC-conjugated antibody through control experiments.

2. **Inconsistent Results:** Results are inconsistent between experiments or replicates.

   Troubleshooting:

   - Ensure that all reagents are properly stored and handled according to recommended protocols.
   - Standardize the experimental procedure and adhere to the same protocols each time.
   - Check the stability of the apoptosis inducing agent and prepare fresh stock of solution-suspension if needed.
   - Use controls, including positive and negative controls, to validate the consistency of the staining.

3. **Poor or Inconsistent Staining Intensity:** Staining intensity may vary from sample to sample or within a sample.

   Troubleshooting:

   - Optimize antibody concentrations and incubation times for Annexin V and the APC-conjugated antibody.
   - Ensure proper fixation and permeabilization to allow reagents to access their targets.
   - Maintain consistent staining conditions across samples.
   - Verify that the APC-conjugated antibody is functioning well in separate experiments.
   - Ensure that reagents are stored and handled appropriately, following recommended protocols to maintain their quality.
   - Standardize the entire experimental procedure, adhering to the same protocols each time.
   - Perform staining controls, positive and negative controls, in every experiment to validate the consistency of the staining protocol.

4. **Loss of Annexin V Binding:** Annexin V staining might be inconsistent or absent.

   Troubleshooting:

   - Optimize Annexin V incubation time to achieve the best binding.
   - Ensure that the cells are properly permeabilized after fixation to allow Annexin V to access phosphatidylserine.
   - Verify that the cells are not over-fixed, as this could affect Annexin V binding.
   - Avoid over-fixation, which could hinder Annexin V binding.

5. **Antibody Background:** The APC-conjugated antibody may exhibit nonspecific binding or high background fluorescence.

   Troubleshooting:

   - Optimize antibody concentration and incubation time to achieve the best signal-to-noise ratio.
   - Use appropriate controls to identify and quantify nonspecific binding.

6. **Antibody Incompatibility or Cross-Reactivity:** The APC-conjugated antibody may have cross-reactivity or incompatibility with other reagents.

   Troubleshooting:

   - Choose primary and secondary antibodies that are specific for the targets and have minimal cross-reactivity.
   - Perform control experiments using individual primary antibodies to confirm specificity.

7. **Compensation Issues:** Compensation is incorrect, leading to inaccurate interpretation of data.

   Troubleshooting:

   - Perform compensation controls using single-stained cells or beads labeled with each fluorochrome.
   - Adjust compensation settings on the flow cytometer software to accurately separate the emission spectra.

8. **Over-Fixation or Under-Fixation:** Fixation might be too strong or too weak, affecting subsequent staining.

Troubleshooting:

- o Optimize fixation time and concentration to achieve the desired preservation of cellular morphology and protein localization.
- o Perform pilot experiments with varying fixation conditions and assess their impact on staining outcomes.

9. **Blocking Agent:** When using Annexin V, which is a protein itself, there could be concerns about potential cross-reactivity or interactions between Annexin V and common blocking agents like BSA.

Troubleshooting:

- o Check for compatibility between Annexin V and the chosen blocking agent. Investigate potential cross-reactivity or interactions.
- o Consider using alternative blocking agents that are known to be compatible with Annexin V.

10. **Antibody Staining Order:** The order of staining with Annexin V and the APC-conjugated antibody might impact the staining outcomes due to potential interactions between reagents.

Troubleshooting:

- o Perform pilot experiments to optimize the staining sequence. Assess whether certain antibodies are sensitive to the presence of other reagents.
- o Determine the best order of staining through systematic testing.

11. **Optimization:** Variability in staining outcomes may arise due to inadequate optimization of fixation duration, permeabilization, blocking, and antibody concentrations.

Troubleshooting:

- o Systematically optimize each step of the protocol for your specific cell type and reagents.
- o Perform a series of experiments to identify the best conditions for fixation, permeabilization, blocking, and antibody staining.

12. **Wash Steps:** Inadequate washing between staining steps can lead to elevated background noise and nonspecific binding.

Troubleshooting:

- o Ensure thorough washing after each staining and blocking step to remove unbound reagents.
- o Optimize the number of washes and the volume of wash buffer used.

13. **Instrument Compatibility:** Variability in instrument sensitivity to different fluorochromes can affect the detection of fluorescence emitted by PI, Annexin V, and APC.

Troubleshooting:

- o Verify that your flow cytometer is capable of detecting the fluorescence of all relevant fluorochromes.
- o Perform controls to ensure proper instrument settings for accurate detection.
- o To stain additional proteins, please make sure that the fluorophore conjugated to the additional protein differs from those previously used and possesses a distinct excitation wavelength. Additionally, ensure compatibility of the new fluorophore with the flow cytometer.

14. **Drugs with autofluorescence properties**: Overlap between doxorubicin and PI emission spectra leading to inability to segregate red fluorescence signals:

Troubleshooting:

- o Utilize flow cytometer with capability for fine-tuned emission filtration and wavelength discrimination to spectrally separate doxorubicin (590 nm peak) from PI emission (617 nm peak). Confirm capability to distinguish signals through single-stain controls.
- o Use PI variants with larger Stokes shifts between excitation and emission peaks to minimize spectral overlap. Consider PI choices like propidium iodide-enrollment (Ex. 535 nm/Em 617 nm).
- o Select an alternative apoptosis detection dye without emission overlap with doxorubicin, like 7-AAD (546 nm Em.) or DyeCycle Violet (457 nm Em.).

- For fixation-permeabilization assays, extract doxorubicin by alcohol treatment prior to analysis to eliminate native fluorescence.
- Use "background cell" group treated with doxorubicin but unstained. The background cell group would be useful during flow cytometry analysis for setting background subtraction thresholds based on doxorubicin's autofluorescence.

3. Results

After triple staining the cells with annexin V-FITC/PI and APC-*anti*-CD44/CD24 staining, we found that doxorubicin treatment initiates the apoptosis in MDA-MB-231 cells (Fig. 1A) compared to untreated controls (Fig. 1B). The late apoptotic population increased from 0.75 % in the control to 4.40 % in doxorubicin treated cells. Similarly, the early apoptotic population surged from 10.8 % (Fig. 1B) to 38.8 % (Fig. 1D) with treatment. Conversely, the healthy cell population declined from 87.9 % (Fig. 1B) to 52.9 % (Fig. 1D) with doxorubicin exposure (Fig. 1).

Doxorubicin also altered CD24 (Fig. 2A) and CD44 (Fig. 2B) expression levels. Total cellular CD24 was slightly higher in doxorubicin-treated cells compared (Fig. 3C) to control (Fig. 4C). Alterations in CD24-APC protein expression was marginal between doxorubicin-treated (Fig. 3D) and control cells (Fig. 4D) across necrotic, late apoptotic, and healthy cell populations. Notably, CD24 expression was significantly reduced early apoptotic compared to the untreated cells (control) (Fig. 2A). Conversely, the highest CD44 expression was observed in early apoptotic cells for both control and doxorubicin treatment (Fig. 2B). Total cellular CD44 was

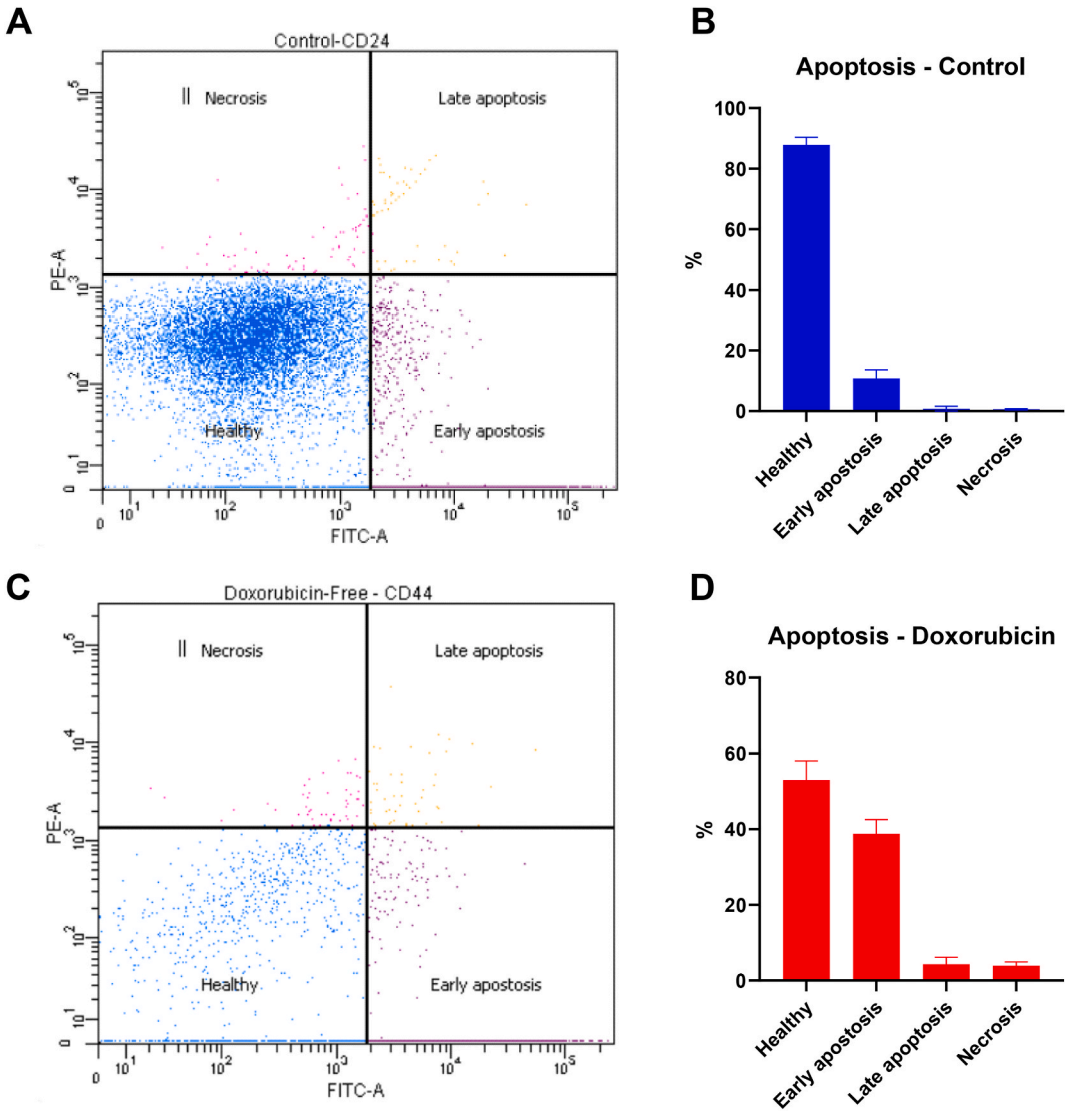

Fig. 1. Annexin V-FITC/PI analysis of MDA-MB-231 breast cancer cells treated with 1 μM doxorubicin for 72 h and stained for surface CD44 and CD24 expression using APC-*anti*-CD44 and APC-*anti*-CD24 antibodies. Quadrants showing Annexin V/PI fluorescence are numbered as follows: Healthy cells (Annexin V-/PI-), Early apoptotic (Annexin V+/PI-), Late apoptotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+) cells.

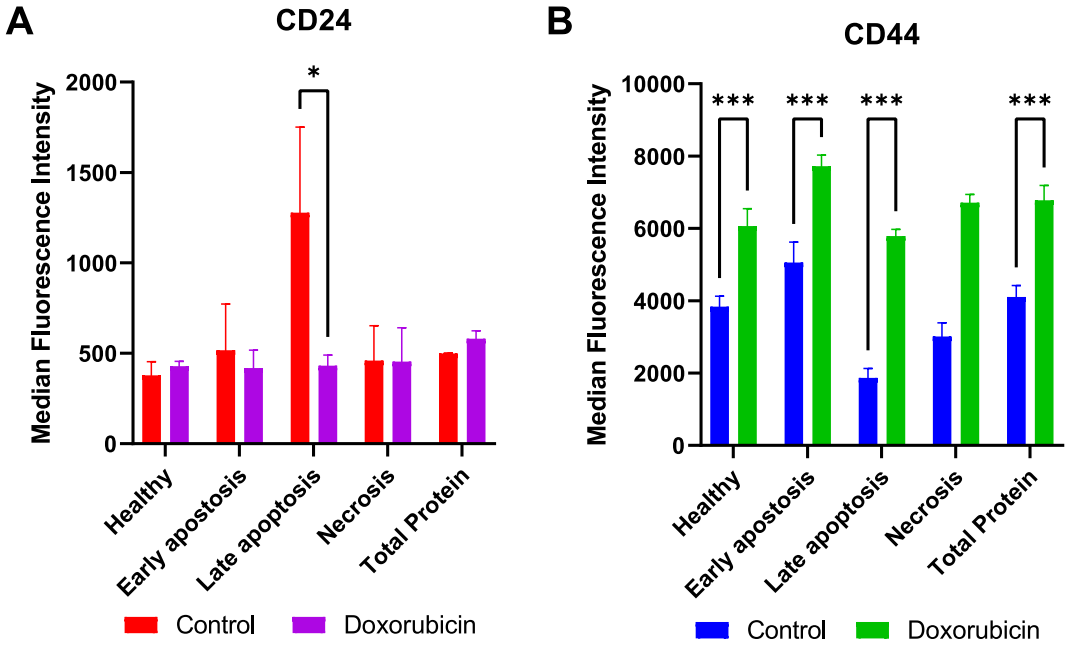

Fig. 2. MDA-MB-231 breast cancer cells treated with 1 μM doxorubicin for 72 h and stained for surface CD24 (A) and CD44 (B) expression among different apoptosis subpopulations. *$p < 0.05$, ***$p < 0.001$.

significantly higher in doxorubicin-treated cells compared (Fig. 3A) to control (Fig. 4A). Doxorubicin increased CD44-APC expression in necrotic, late apoptotic, and early apoptotic cells (Fig. 3B) compared to controls (Fig. 4B).

4. Discussion

Doxorubicin is a chemotherapeutic agent commonly used to treat breast cancer. It works by intercalating DNA and inhibiting topoisomerase II, leading to DNA damage and apoptosis in cancer cells [12]. Doxorubicin treatment alter the expression of cell surface proteins like CD44 and CD24, both of which have been associated with breast cancer stem cells. In this study, an in-depth investigation was conducted to explore the effects of doxorubicin treatment on CD44/CD24 expression in MDA-MB-231 triple negative breast cancer cells, focusing on distinct subpopulations undergoing apoptosis. This study focuses specifically on the MDA-MB-231 line as a proof-of-concept example.

Fig. 2 A shows that CD24 expression was significantly ($p < 0.05$) smaller in the late apoptotic stage compared to control. While the opposite happened for CD44 where it was very significantly ($p < 0.001$) increased in all healthy and apoptotic subpopulations. Also, our findings align with the established characterization of MDA-MB-231 cells, specifically the presence of the CD44 protein and the absence of CD24 protein in the control cell group [8]. This demonstrates the utility of this technique to quantify apoptosis induction and concurrently monitoring protein expression changes in breast cancer cells.

Among the treated group, our results showing CD44 enrichment and CD24 downregulation in MDA-MB-231 cells after doxorubicin exposure, which are consistent with previous studies investigating these markers. For instance, Li et al. found that doxorubicin treatment increased the CD44+/CD24-population and expression of stemness genes in MCF-7 and MDA-MB-231 breast cancer cells [13]. Also, our results aligned with the realization that doxorubicin increases CSCs which leads to building up drug resistance [14]. Additionally, it was reported that doxorubicin selected for a CD44+/CD24-/low subpopulation in MDA-MB-468 cells, which exhibited CSC properties like enhanced mammosphere formation [15]. The expansion of the CD44+/CD24-phenotype implicates the outgrowth of cells with enhanced stemness and tumor-initiating potential, a proposed mechanism of acquired therapeutic resistance [16]. Potential strategies may involve combining doxorubicin with agents that suppress CD44, stimulate CD24 re-expression, or selectively eliminate cells exhibiting a CD44+/CD24-phenotype through antibody-drug conjugates [17–20].

Our findings have important clinical implications. High CD44 and low CD24 expression is associated with enhanced invasion, metastasis, therapeutic resistance, and poor prognosis in breast cancer patients [21,22]. Therefore, the outgrowth of CD44+/CD24-cells after chemotherapy observed here and in other studies [13] likely contributes to recurrence and mortality. Targeting CD44 or the CSC population may help overcome resistance [23]. For example, combining doxorubicin with CD44 inhibition improves efficacy in preclinical models [24]. Furthermore, high CD44 and low CD24 in circulating tumor cells predicts increased risk of metastasis and death in patients [25]. Our quantitative apoptosis-protein profiling approach provides a tool for identifying strategies to suppress outgrowth of resistant CD44+/CD24-cells.

Furthermore, the increase in CD44 expression and enrichment of CD44+/CD24-cells induced by doxorubicin may arise through several potential mechanisms. Doxorubicin could selectively kill CD44-/low cells while promoting the survival and outgrowth of

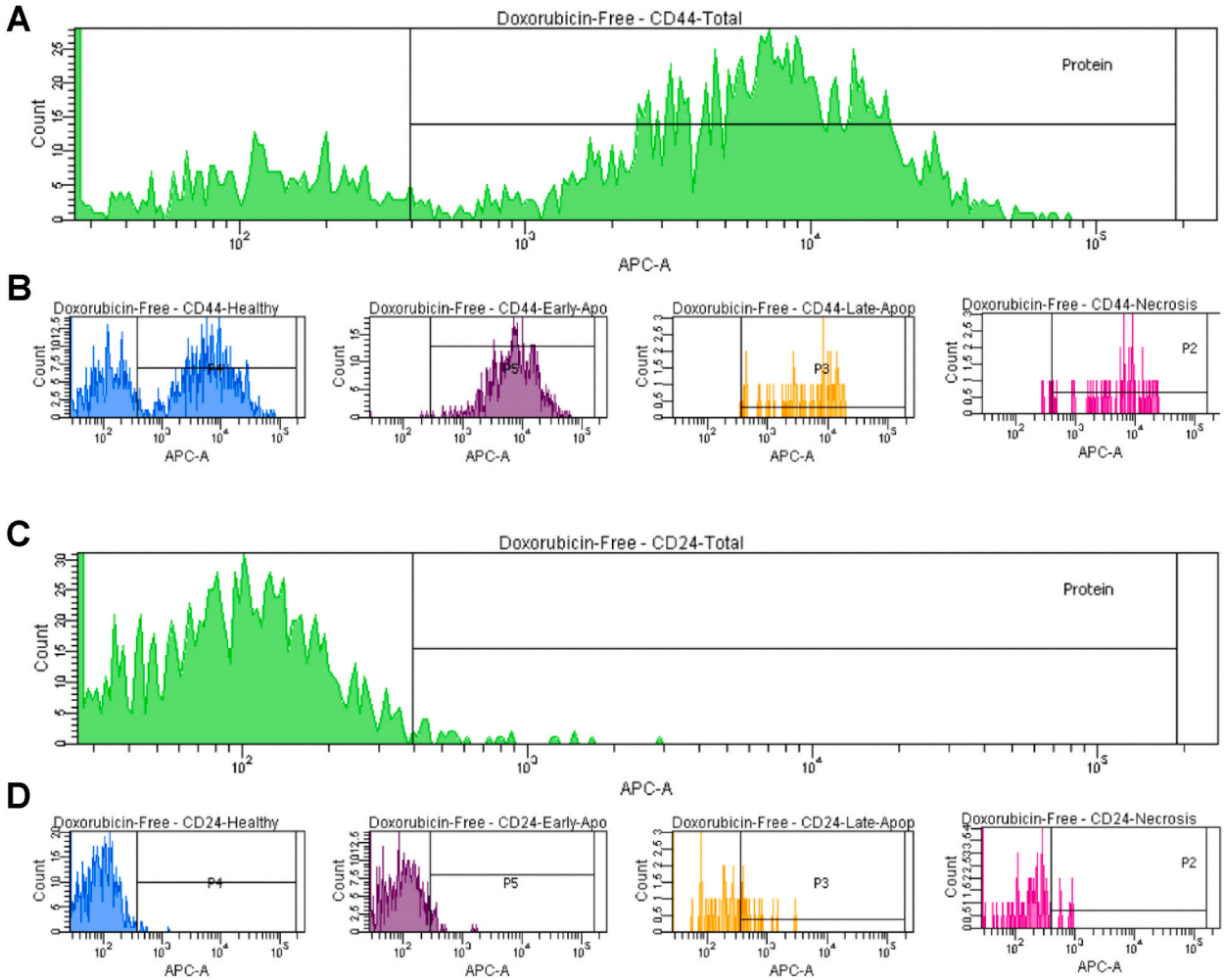

Fig. 3. Histogram of MDA-MB-231 breast cancer cells treated with 1 μM doxorubicin for 72 h and stained for surface CD44 (A) and CD24 (C) expression among different apoptosis subpopulations (B and D).

innately resistant CD44+/CD24-cells [13]. Additionally, doxorubicin treatment can stimulate reactive oxygen species production and activation of stress response pathways like Nrf2, which have been linked to upregulating CD44 and stemness-associated genes [26,27]. Doxorubicin may also enrich for CD44+/CD24-cells by promoting epithelial-to-mesenchymal transition (EMT), a process associated with gain of stem cell properties [28]. Finally, chemotherapy can induce de-differentiation of non-CSC populations into CSCs, as evidenced by conversion of $CD24^{+}$ into CD44+/CD24-cells [29]. The enrichment of CD44 in both apoptotic and healthy cell populations lends support to the latter two proposed mechanisms of action for doxorubicin. Further investigation delineating the predominant mechanism(s) underlying doxorubicin-mediated increases in CD44 expression and expansion of the CD44+/CD24-phenotype is warranted. While the specific pathways have yet to be fully elucidated, collectively these studies point to multiple means by which doxorubicin treatment can modulate the phenotypic profile and stem-like state of cancer cell subpopulations. Moreover, there are several signaling pathways and stress response mechanisms that have been implicated in regulating CD44 expression and cancer stemness, including NF-kB signaling can upregulate CD44 transcription and maintains the CSC phenotype [30]. Also, HIFs activated by hypoxia can bind the CD44 promoter and increase transcription [31]. Furthermore, Nrf2, a mediator of antioxidant responses, upregulates CD44 and other stemness genes when activated by oxidative stress [32]. And TGF-beta signaling through Smad and non-Smad pathways increases CD44 and promotes EMT [33], a process linked to gaining stem-like properties. Also, MAPK, PI3K/Akt, Wnt/β-catenin pathways have been associated with increased CD44 expression and stemness in various contexts [34]. Additional research into the differential impacts on healthy versus apoptotic cells will help clarify the role of stem cell marker upregulation in treatment resistance versus treatment-induced cell death.

Our multiparametric flow cytometry analysis demonstrates that doxorubicin promotes stem-like CD44+/CD24-cells, supporting the view that this population mediates drug resistance and aggressive tumor regrowth. By connecting the kinetics of apoptotic cell death to associated protein signaling, our approach provides a robust methodology for elucidating the molecular mechanisms underlying chemoresistance. These results have important implications for improving breast cancer therapy, as the enrichment of

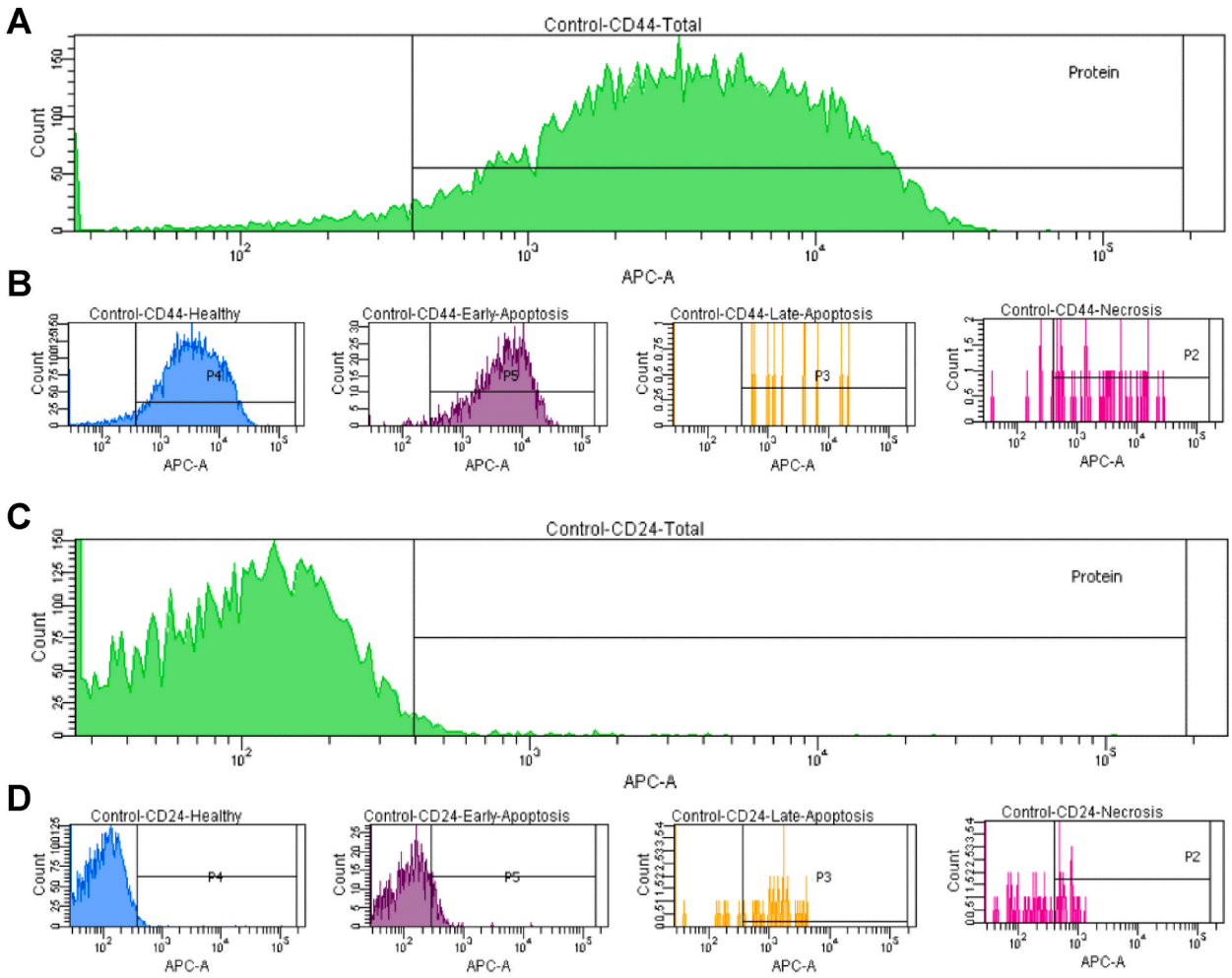

Fig. 4. Histogram of MDA-MB-231 breast cancer untreated cells and stained for surface CD44 (A) and CD24 (C) expression among different apoptosis subpopulations (B and D).

treatment-resistant cancer stem cells after doxorubicin highlights the need to target this population to prevent recurrence. More broadly, our integrated apoptosis and protein profiling technique could be applied to patient-derived xenograft models or clinical samples to reveal signaling pathways and therapeutic targets that overcome resistance. Future drug discovery and delivery investigations into new drugs, synergistic combinations, and delivery systems should focus on increasing the ratio of cancer stem cells undergoing apoptosis while decreasing this ratio in healthy cell subpopulations. These findings lay the groundwork for identifying optimized therapies that selectively target treatment-resistant cancer stem cells.

5. Conclusion

Alterations in protein expression provide insights into signaling pathways involved in apoptotic responses. This study focuses specifically on the MDA-MB-231 line as a proof-of-concept example. Employing triple staining involving Annexin V-FITC/PI and APC-conjugated antibodies yields robust quantitative data pertaining to both apoptosis and protein expression dynamics within well-defined cell populations. Appropriate filter selection and compensation controls are necessary for correct interpretation. This powerful multiparametric approach has broad applications for elucidating mechanisms of apoptotic response to anti-cancer treatments in diverse cellular. Further optimization of antibody panels enables in-depth profiling of the signaling networks that regulate apoptosis and therapeutic resistance. Overall, this comprehensive protocol presents a highly valuable and adaptable tool for advancing cell death research with implications for enhancing cancer therapy, fostering a promising avenue for progress.

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Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Salah Abdalrazak Alshehade: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Hassan A. Almoustafa:** Writing – review & editing, Resources, Investigation, Conceptualization. **Mohammed Abdullah Alshawsh:** Writing – review & editing, Project administration, Conceptualization. **Zamri Chik:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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