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Refined annexin V/PI apoptosis protocol to minimize false-positive necrotic cells

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ABSTRACT

Programmed cell death (PCD) is a fundamental biological process required for tissue homeostasis and the removal of damaged or infected cells. Apoptosis, one of the most studied PCD modalities, is routinely assessed using the Annexin V/propidium iodide (PI) assay. However, the conventional Annexin V/PI protocol frequently produces false-positive necrotic signals due to membrane damage caused during cell harvesting and handling. This artifact compromises the metrological accuracy of necrosis quantification, limiting assay standardization in cancer research and potentially leading to the premature exclusion of promising anticancer compounds during in vitro screening. To address this issue, we developed a modified Annexin V/PI protocol designed to improve the reliability of necrosis detection. In the optimized approach, PI is added directly to the culture medium prior to cell harvesting, allowing uptake exclusively in cells with truly compromised membrane integrity. This strategy effectively reduces handling-induced artifacts and provides a more accurate evaluation of the membrane-compromised population. Precise discrimination between apoptotic and necrotic cells is essential for standardized cellular assays and for the accurate interpretation of drug-induced cytotoxic effects. Our revised protocol enhances accuracy, reproducibility, and robustness of apoptosis assessment, particularly in fragile cell systems, while remaining fully compatible with commercially available Annexin V/PI detection kits.

Introduction

Programmed cell death (PCD) is an essential biological phenomenon, regulating embryogenesis, tissue homeostasis, immune response, cell turnover, response to cellular stress and elimination of damaged or infected cells [1]. Despite its physiological function, it is also involved in the pathogenesis of several diseases, including cancer, neurodegenerative and autoimmune disorders, where both the excessive and the reduced programmed cell death may contribute to disease progression [2]. PCD can be classified according to its morphological features, biological context and triggering mechanisms. Over the years, researchers have identified many different types of PCD, operating through interconnected molecular signaling pathways with extensive crosstalk [3]. The main types of PCD known today include apoptosis, autophagic cell death, lysosomal cell death, mitoptosis, paraptosis, pyroptosis, NETosis, necroptosis, ferroptosis and many others [4]. Among these mechanisms, apoptosis is one of the most widely studied. First described by Kerr in 1972 [5], apoptosis is a fundamental form of programmed cell death. This process, driven by a caspase cascade, leads to the elimination of cells without typically provoking inflammatory responses [6]. Apoptosis is further divided into intrinsic and extrinsic pathways, distinguished by their upstream signals. The extrinsic pathway is triggered by the activation of death receptors on the plasma membrane [7], whereas the intrinsic pathway primarily arises from a wide range of non-receptor-mediated stimuli [8]. Regardless of the type of pathway activated, apoptosis is characterized by the blebbing of the cell membrane, chromatin condensation, DNA fragmentation, cell shrinkage, and detachment from the extracellular matrix [9].

Among the different methods for apoptosis detection, Annexin V binding assay is an established technique for the identification of

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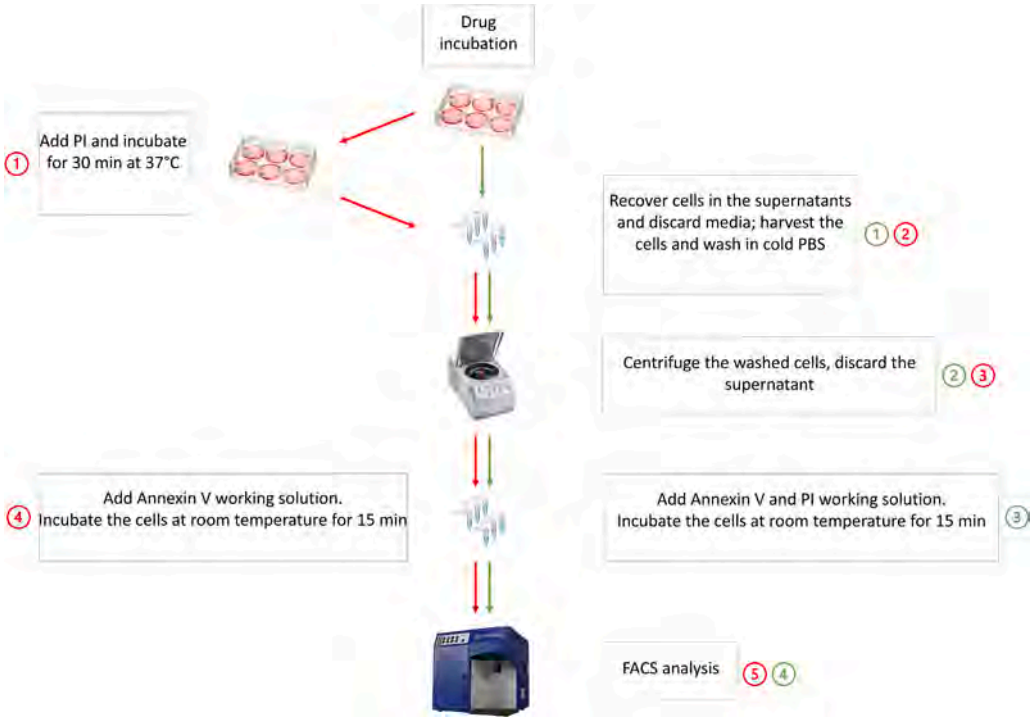

Fig. 1. Schematic representation of apoptotic protocols used. In red is indicated the proposed methodology (modified method) while in green the classical apoptosis protocol.

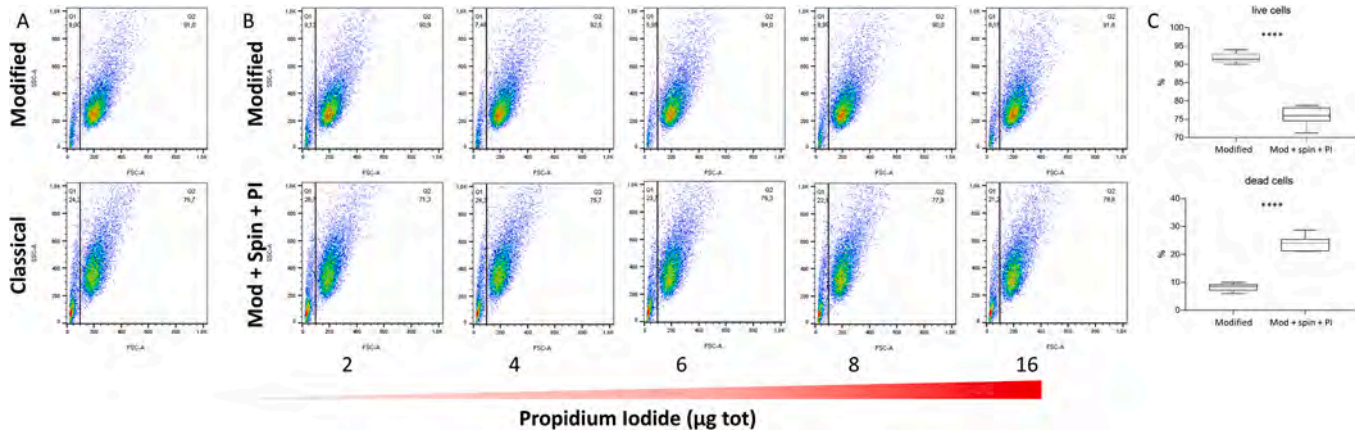

Fig. 2. (A) FACS histograms of untreated SK-LU−1 cells stained with 4 µL PI (4 µg total in 1.5 mL of medium per well) either added directly to the culture medium (top graph) or added after one PBS wash, centrifugation, and resuspension (bottom graph). (B) FACS histograms of untreated SK-LU−1 cells stained with increasing PI concentrations (2, 4, 6, 8, and 16 µg total in 1.5 mL of medium per well), added directly to the medium (top graphs) or after a PBS wash, centrifugation, and resuspension (bottom graphs). (C) Bar plot comparing PI-positive cells between centrifuged and not centrifuged conditions (Paired t-Test, n = 5 for each condition, both p-value< 0.0001).

apoptotic cells and their quantification [10]. Annexin V is a calcium-dependent phospholipid-binding protein that has a strong affinity for phosphatidylserine (PS), one of the glycerophospholipids that constitute the cell membrane. In healthy cells, PS is retained in the inner (cytosolic) leaflet of the membrane, whereas during apoptosis it is translocated to the extracellular side [11]. The exposure of PS on the outer leaflet can be detected using fluorescently labeled Annexin V [12]. Annexin V is commonly used together with propidium iodide (PI), a nucleic acid-binding dye that helps to distinguish different stages of cell death [13]. Because PI can enter cells only when membrane integrity is compromised, it stains dead and late apoptotic cells. Once inside, PI intercalates with nucleic acids and emits red fluorescence [14]. Since necrotic cells have ruptured membranes, Annexin V can also stain them. The co-staining with PI serves the purpose of distinguishing necrotic cells from apoptotic ones, since PI enters necrotic cells but it is excluded from early apoptotic ones [15]. The binding of Annexin V can be assessed using commercially available kits and visualized via flow cytometry, fluorescence microscopy or bright-field microscopy.

The first study to describe the use of Annexin V in combination with a membrane-impermeant dye for the flow cytometric detection of apoptosis was conducted by G. Koopman et al [16]. , who employed ethidium bromide to stain cells with compromised membrane integrity. Subsequently, in 1995, Vermes et al [17]. refined this approach by substituting propidium iodide for ethidium bromide, thereby laying the foundation for most commercially available Annexin V/PI apoptosis detection kits. In the standard protocol, Annexin V/PI staining is performed after cell harvesting, followed by centrifugation, washing with PBS, and resuspension in the binding buffer containing the two dyes.

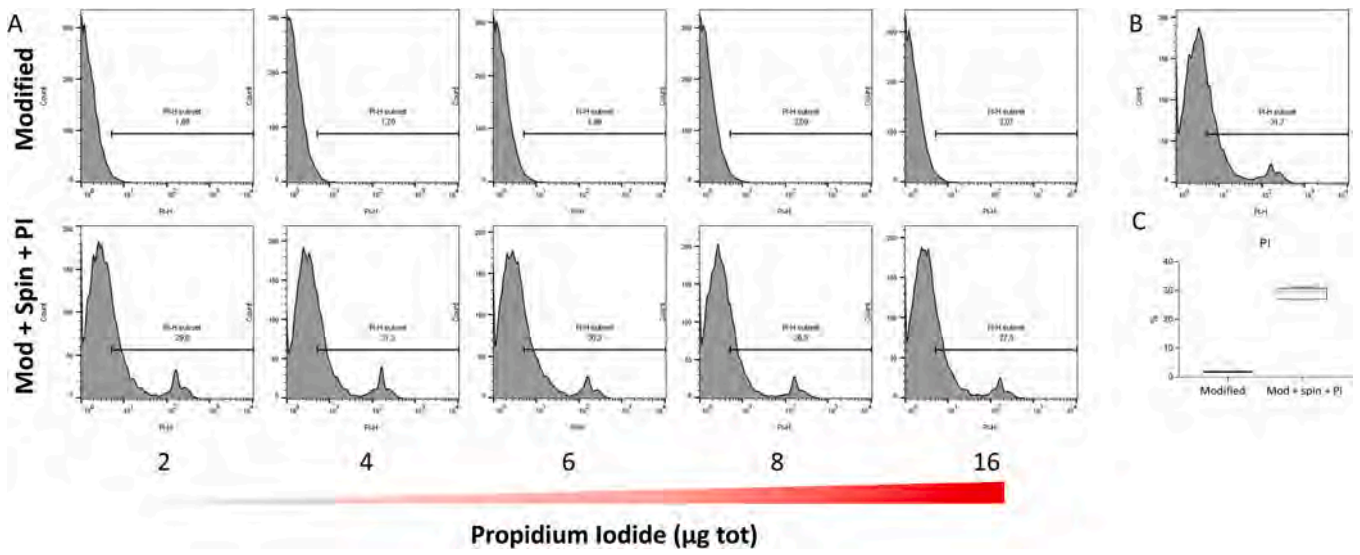

Fig. 3. (A) FACS histograms of SK-LU−1 samples from the previous experiment, stained with increasing total PI concentrations (2, 4, 6, 8, and 16 µg in 1.5 mL of medium per well), either added directly to the culture medium (top graphs) or applied after one PBS wash, centrifugation, and resuspension (bottom graphs). (B) FACS histogram of untreated SK-LU−1 cells stained following the classic PI staining protocol. (C) Difference in PI fluorescence intensity between not centrifuged and centrifuged samples. Samples were treated as technical replicates, despite being stained with different PI concentrations (Paired t-Test, $n = 5$ for each condition, p-value < 0.001).

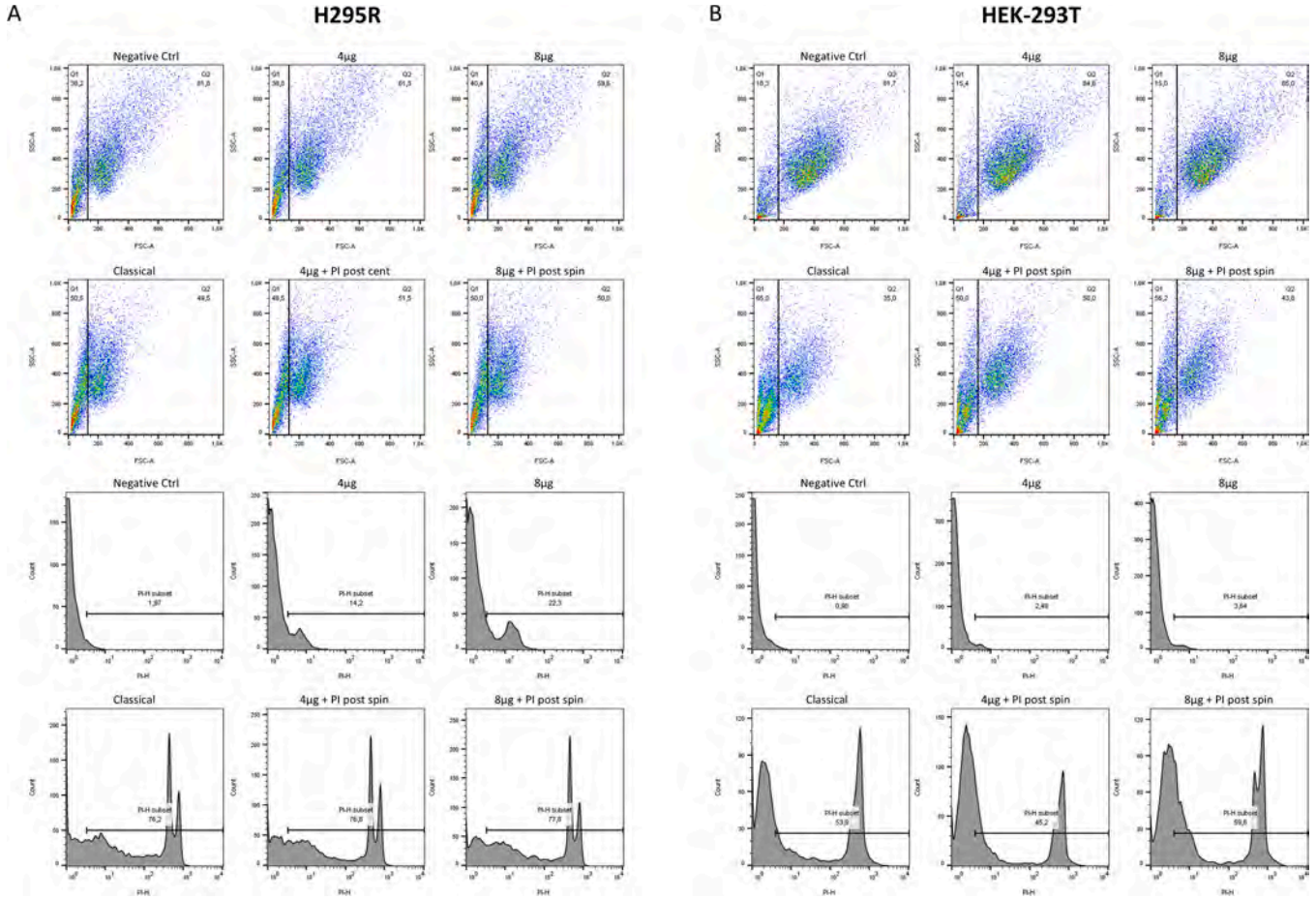

Fig. 4. (A) FACS analysis of H295R cells showing population distributions (top) and PI fluorescence intensities (bottom) after staining with 4 or 8 µg PI added directly to the culture medium or applied using the classic staining protocol following centrifugation. (B) FACS analysis of HEK-293T cells showing population profiles (top) and PI fluorescence intensities (bottom) under the same staining conditions (4 or 8 µg PI added directly to the medium vs. classic protocol after centrifugation).

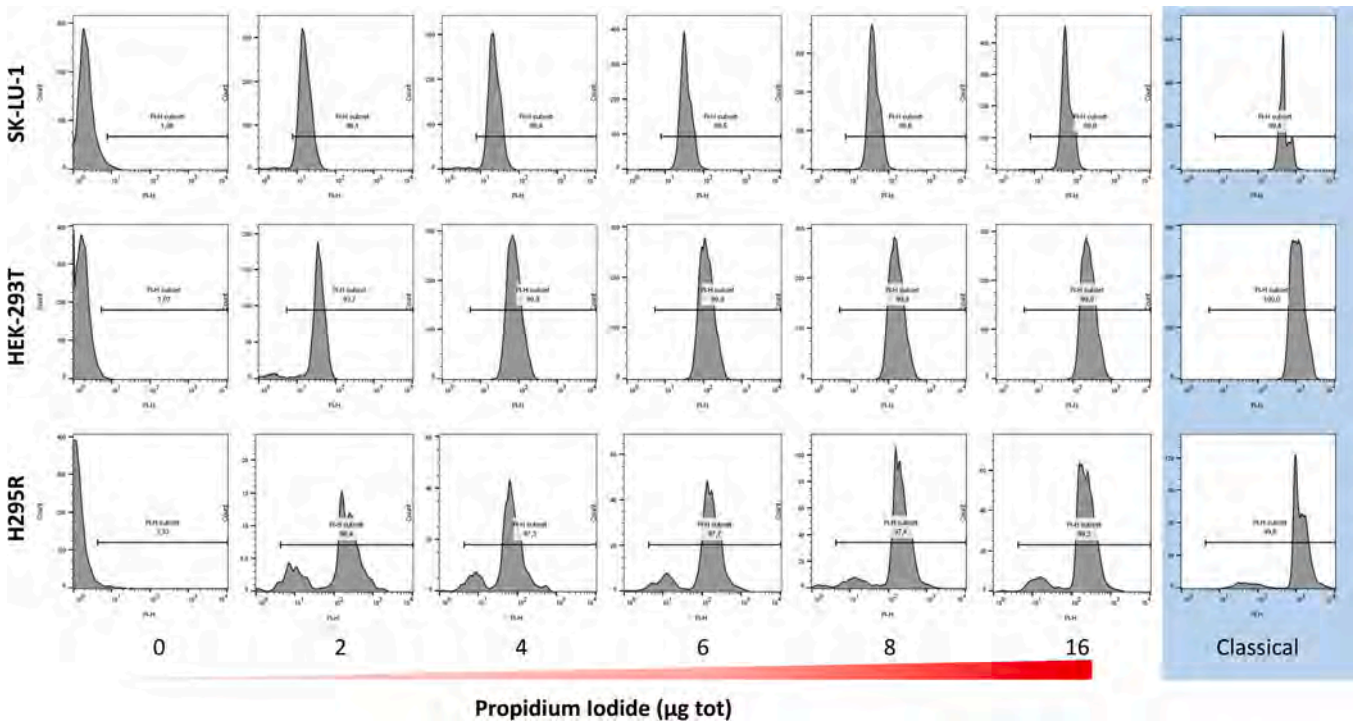

Fig. 5. FACS analysis showing PI fluorescence intensities in SK-LU−1, HEK-293T, and H295R cells treated overnight with 50% DMSO and stained with increasing PI concentrations (0, 2, 4, 5, 8, and 16 µg in 1.5 mL of medium per well) added directly to the culture medium and compared with the classical protocol.

Despite its widespread use, the standard Annexin V/PI protocol presents methodological limitations that can compromise the accuracy of necrosis quantification. Indeed, based on literature [18] and our experimental experience, this conventional Annexin V/PI protocol often results in a substantial number of false-positive necrotic events. These artifacts are primarily due to PI uptake by cells that become damaged during sample handling, a problem that is particularly evident with more fragile cell types. Accurate discrimination between apoptotic and necrotic populations is essential for the reliable interpretation of drug-induced cytotoxic effects, especially when fragile cell types are analyzed.

Here, we present a modified Annexin V/PI protocol that enhances the accuracy of necrosis assessment compared to conventional methods. In this approach, PI is added directly to the culture medium, allowing PI uptake selectively in cells that are compromised by the experimental condition (e.g., drug exposure) and not by the sample processing steps. This pre-staining strategy minimizes handling-induced membrane damage and reduces artificial PI incorporation. In addition, our results indicate that this modification is fully compatible with commercially available Annexin V/PI apoptosis detection kits.

Materials and methods

Cell lines and culture conditions. All cell lines were cultured at 37°C with 5% CO2 according to American Type Culture Collection (ATCC) instructions. Media and supplements were purchased from Euroclone unless otherwise stated. H295R cells were grown in DMEM: F12 50:50 medium (Gibco: Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 2.5% NuSerum (Corning, #355100, Thermo Fisher Scientific, Waltham, Massachusetts, USA), penicillin/streptomycin (Gibco) and ITS Premix (Corning #354350). HEK-293T and SK-LU−1 cells were grown in DMEM medium (Gibco: Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% Fetal Bovine Serum (FBS; Thermo Fisher Scientific, Waltham, Massachusetts, USA), penicillin/streptomycin (Gibco). Unless indicated, all experiments were conducted in the absence of serum [19]. Cell lines were periodically tested for mycoplasmas and authenticated by genetic profiling through polymorphic short tandem repeat loci with the PowerPlex® 16 System (Promega, Madison, Wisconsin, USA) and Applied Biosystems 3130 genetic Analyzer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Regular testing and authentication ensured traceability and identity consistency across experimental batches. The cell lines were also treated with the following substances as indicated in the specific paragraphs: Propidium iodide solution (PI, 1 mg/mL) (P4864, Sigma-Aldrich, Darmstadt, Germany); Dimethyl sulfoxide (DMSO) (D8418, Sigma-Aldrich, Darmstadt, Germany); cis-Diammineplatinum(II) dichloride (CDDP) (232120, Sigma-Aldrich, Darmstadt, Germany).

FACS. The FACS acquisition was done using BD FACS Celesta (Becton Dickinson Biosciences, Franklin Lakes, New Jersey, USA). For PI detection, excitation was set at 575 nm and emission was collected at 610 nm, whereas FITC was excited at 488 nm and emission was collected at 530 nm. Each fluorochrome was evaluated by optimizing the laser power so that unstained cells did not fluoresce, and subsequently evaluating specific fluorescence in appropriate positive controls. When both channels were acquired, compensation was performed using the specific BD FACSDiva™ tool software on single-stained positive controls. Since the aim was to evaluate necrotic cells, no gates were applied to the results except to exclude very small debris present near the intersection of the axes. Because apoptotic and necrotic cells often show atypical physical parameters, we deliberately minimized gating to avoid unintentionally excluding these altered populations. This approach also ensures that the proposed method remains compatible with, and can be readily adapted to, cell type-specific or optimized analysis settings used by different laboratories. Data analysis and figures generation were performed using the FlowJo™ Software (FlowJo™ Software Version 10, Ashland, USA).

Image staining and count. SK-LU−1, HEK-293T, and H295R cells were seeded in 96-well plates at a density of 5×10^4 cells per well. For imaging using the proposed modified method, cells were stained with propidium iodide (0.13 µL of PI stock solution per 50 µL, corresponding to a final amount of 4 µg in 1.5 mL) and CellTracker™ Green CMFDA

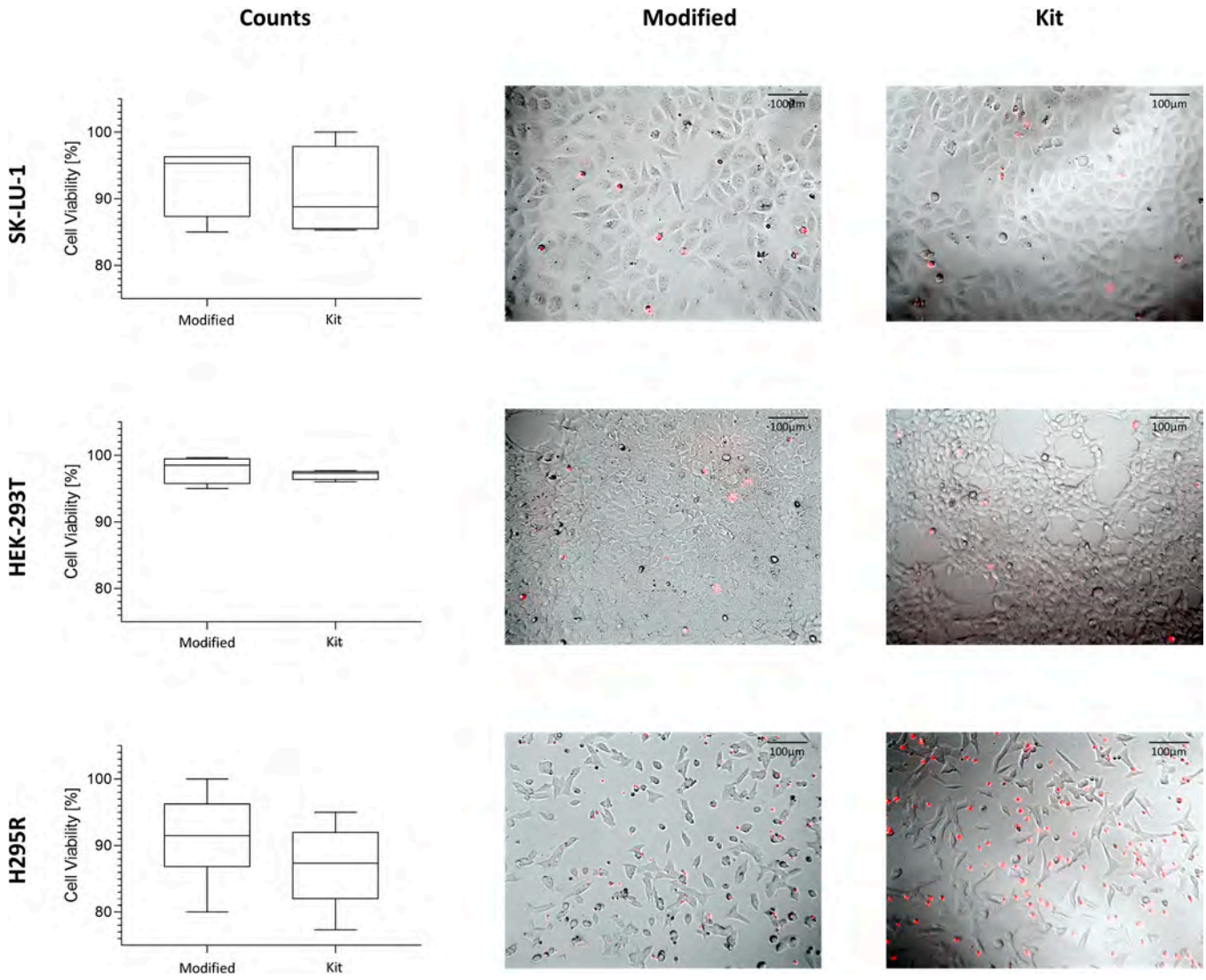

Fig. 6. Cell viability quantification using the proposed method (indicated as "modified" in the bar plots) and the LIVE/DEAD™ Cell Imaging Kit (indicated as "kit"). For each condition four independent wells were counted in technical triplicate. The bar plots on the left show the percentage of viable cells for each cell line, assessed using PI added directly to the culture medium (modified) or using the commercial kit. Cell viability was calculated from the ratio of viable to dead cells. Representative merged images on the right show brightfield and red fluorescence (PI staining) for each analyzed cell line (10X magnification).

(C2925, Invitrogen), a fluorescent dye for viable cells. CellTracker staining was performed according to the manufacturer's instructions. The proposed method was compared with the commercial LIVE/DEAD™ Cell Imaging Kit (R37601, Invitrogen), which assesses cell viability based on intracellular esterase activity and plasma membrane integrity. The LIVE/DEAD™ assay was performed according to the manufacturer's instructions. Images were acquired using a Leica DMIRE2 microscope at 10 × magnification. For each field, brightfield, green fluorescence, and red fluorescence images were collected and subsequently merged using ImageJ software. Cells were counted using the C100 Automated Cell Counter (RWD Life Science, Shenzhen, Guangdong, China). For quantitative analysis, four independent wells were analyzed for each cell line using both the proposed method and the LIVE/DEAD™ Cell Imaging Kit. Cell counting was performed as follows: (i) collection of the well supernatant; (ii) one PBS wash, which was also collected; (iii) centrifugation of the recovered medium at 400–500 g for 5 min; and (iv) detachment of adherent cells using Accutase (Gibco). Cell detachment was performed in serial, one well at a time. For each condition four independent wells were counted in technical triplicate. Results were expressed as percentage cell viability.

Counts and centrifugations. Untreated H295R cells were stained with PI, optimizing the concentration according to cell number. In previous experiments, 1×10^6 cells were stained with 4 µg PI in 1.5 mL of medium; therefore, for dishes containing approximately 25–30 $\times$ 10^6 cells, 120 µg PI in 6 mL of medium was used. After 30 min of incubation at 37°C, the supernatant was collected and the cells were washed once with PBS, which was also recovered to ensure complete cell collection. Cells were then detached using Accutase and resuspended in PBS to obtain a solution with the concentration of 1×10^6 cells/mL, and 18 aliquots of 1 mL were prepared. The number of PI-positive cells in each aliquot was initially quantified using the C100 Automated Cell Counter. Samples were then divided into three groups (n = 6 for each experimental group) and centrifuged at 100, 300 or 500 g. For each sample, the supernatant was collected and the cell pellet resuspended in 1 mL PBS, after which PI-positive cells were quantified again. Subsequently, 2.6 µg PI, which corresponded to 4 µg PI/1.5 mL, was added to each sample, followed by a 30 min incubation at 37°C, and PI-positive cells were counted once more. To evaluate cell loss associated with different centrifugation speeds, the absorbance of each collected supernatant was measured.

Apoptosis analysis. To measure apoptosis, 1×10^6 cells per well were seeded in 6-well plates in the absence of serum; after incubation with the appropriate drug concentration necessary to observe effect on cell line,

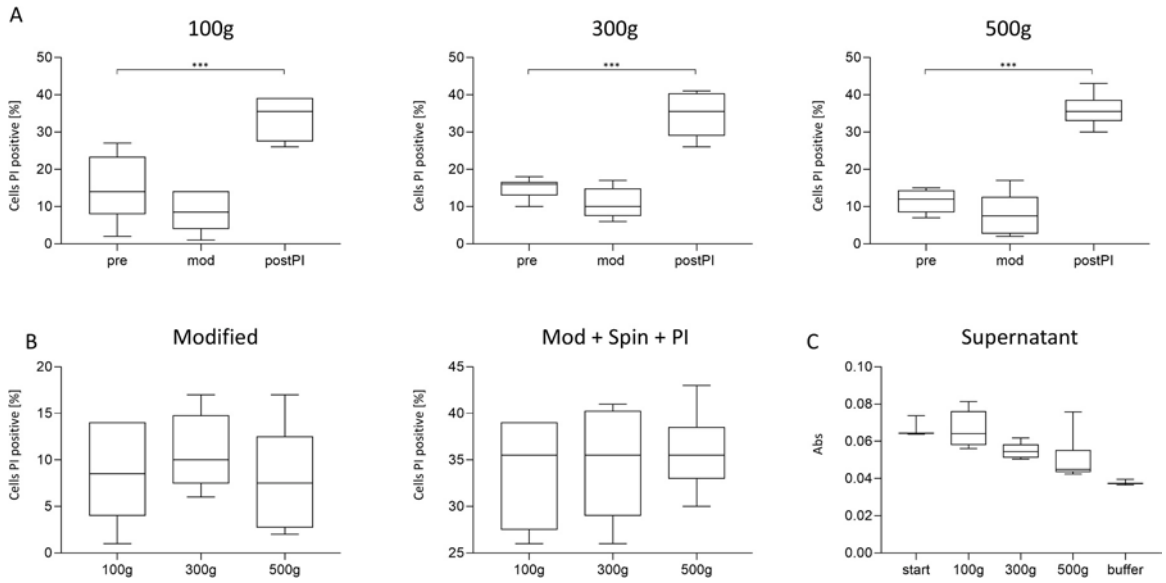

Fig. 7. Evaluation of the effect of centrifugation and centrifugation speed in untreated H295R cells. Cells were stained with PI directly in the medium using the modified protocol and analyzed before and after centrifugation at 100, 300, or 500 g (n = 6 per group). (A) Percentage of PI-positive cells measured before and after centrifugation (indicated as "pre" and "mod"), and after PI addition following the centrifugation (indicated as "mod + spin + PI"). No significant differences were observed before versus after centrifugation at any speed, whereas a significant increase in PI-positive cells was detected when PI was added after centrifugation, independently of centrifugation speed (n = 6 for each condition, all paired t-Test, p-value < 0.01). (B) Comparison of the percentage of dead cells across different centrifugation speeds for both staining approaches, showing no significant differences among centrifugation speed in the two methods. (C) Absorbance of collected supernatants after centrifugation at different speeds, with cell suspensions and resuspension buffer used as positive and negative controls, respectively.

cells were detached with Accutase (cat. #00–4555–56, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stained with Propidium Iodide (P3566 Invitrogen 1 mg/mL) and FITC-Annexin V (A211.01 Vazyme, Nanjing, PRC). FACS analysis was performed with BD FACS Celesta (Becton Dickinson Biosciences, Franklin Lakes, New Jersey, USA) as described previously. For visualization of results, cells without small debris were separated into four quadrants: early apoptotic cells (Annexin positive/PI negative), necrotic cells (Annexin negative/PI positive), late apoptotic cells (Annexin positive/PI positive) and viable cells (Annexin negative/PI negative). The PI working concentration and its specific use in each assay are explicitly indicated in the corresponding experimental descriptions. In selected experiments, PI staining was assessed alone following our modified protocol, described below. The PI was also re-added to the same sample after centrifugation to assess the effect of post-harvesting manipulation on PI uptake.

Modified Protocol (Fig. 1)

1. Add 4 µL of PI stock solution (corresponding to 4 µg of total PI) directly to the culture medium Dose sufficient to stain approximately 1×10^6 cells; adjust as needed depending on cell number and cell line. Incubate for 30 min at 37°C. This step ensures that PI uptake occurs under physiological conditions and prior to any mechanical manipulation.
2. Wash the dish/well with 1X PBS.
3. Harvest cells, referring to specific detachment procedures validated for each cell line or primary cell samples.
4. Centrifuge samples at 400–500 g for 5 min and discard the supernatant.
5. Wash once by resuspending cells in 1X PBS; centrifuge again at 400–500 g for 5 min and discard the supernatant.
6. Prepare 1X Binding Buffer containing only fluorochrome-conjugated Annexin V, according to the manufacturer's recommendations.
7. Resuspend 1–5 $\times$ 10^6 cells/mL cells in 200 µL of 1X Binding Buffer.
8. Incubate for 15 min at room temperature protected from light.
9. Analyze with flow cytometry.

Results

The widely used "gold standard" for measuring apoptosis and necrosis is the Vermes et al. Annexin V/PI FACS assay [17]. It identifies necrotic cells by PI uptake (membrane permeabilization) and apoptotic cells by Annexin V–FITC binding to externalized phosphatidylserine. This simple and elegant method, while reliable in cells that tolerate handling, can overestimate necrosis in membrane-fragile models, sometimes reporting up to ~50% "necrotic" cells despite a largely healthy morphology under the microscope. For this reason, we investigated whether we could modify the method to improve the assessment of necrotic cells, aligning it with what we observe on the plate. To support our findings, we used epithelial cell lines that, based on our experience and as shown by the data presented here, display different levels of resistance to handling and manipulation. Among them, SK-LU−1 showed the highest resistance to manipulation, while HEK-293T and H295R proved to be very sensitive. We propose a change in the standard apoptosis protocol, to achieve a better representation of what we effectively observe, especially in our untreated control cells. This change consists of the addition of PI directly to the medium of the dish/well followed by a 30 min incubation to let the staining occur. In this way, the PI will penetrate and stain only the cells that are truly necrotic and not the ones whose membrane is damaged by the subsequent manipulations that follow cell detachment.

To establish baseline conditions in untreated cells, we used SK-LU−1, which is relatively resistant to handling and therefore serves as a "resistant" model. Fig. 2 compares FACS event populations from untreated SK-LU−1 cells stained either by adding PI directly to the culture medium (modified protocol) or by the classical workflow (PI added after: detachment, PBS wash, centrifugation, and resuspension). Here, we focus on the distribution of acquired events rather than PI fluorescence intensity. Using the black line as a threshold, events to the right correspond to healthy cells, whereas events to the left represent abnormal-sized or dead cells.

In Fig. 2 A, the same sample shows 9% necrotic cells with our method (4 µg PI in 1.5 mL medium per well; Fig. 2 A, upper plot) versus 24.3% with the classical protocol (Fig. 2 A, lower plot). While ~24% necrosis

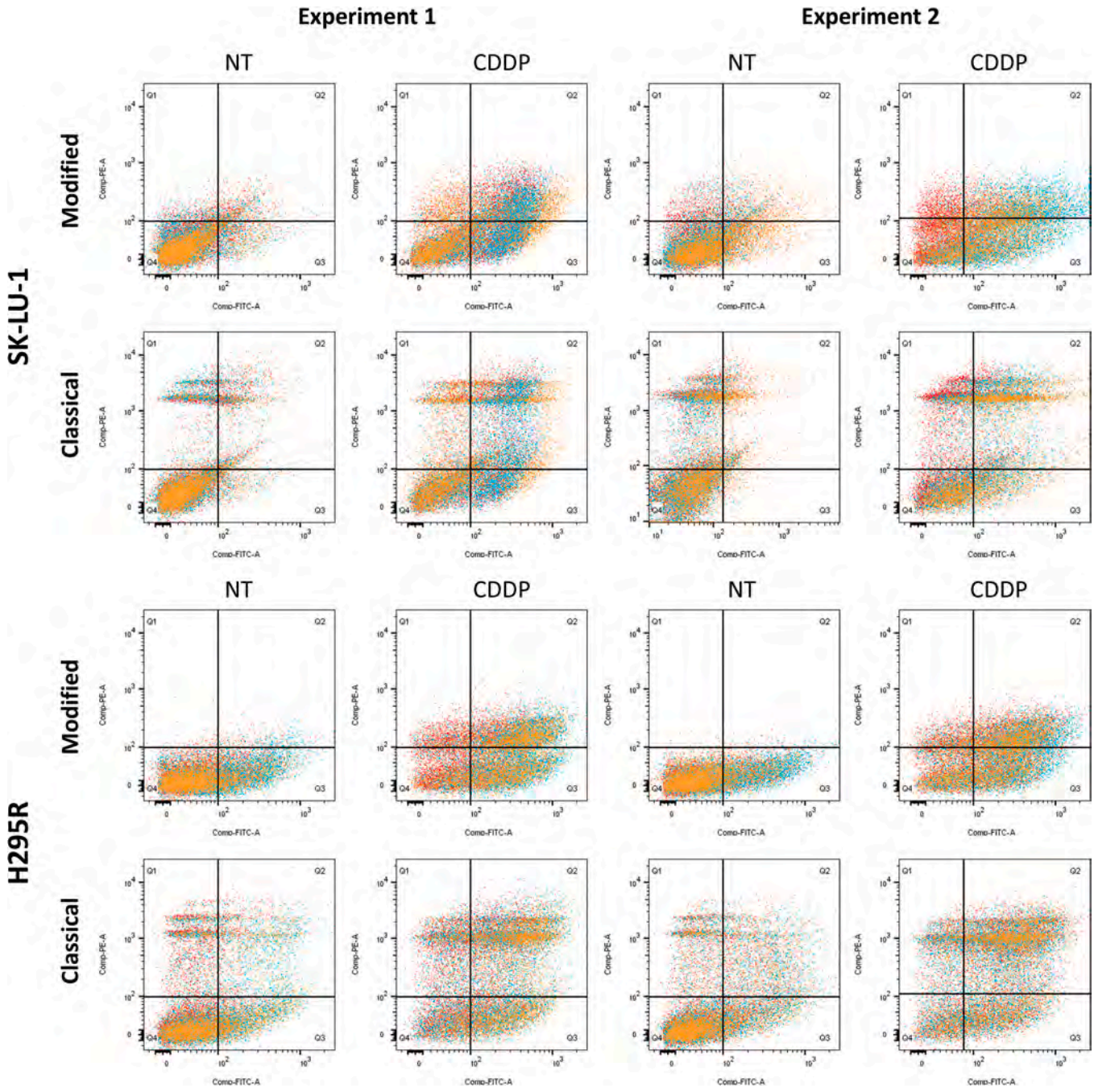

Fig. 8. Apoptosis analysis by FACS in SK-LU−1 and H295R cells treated for 48 h with CDDP (100 μM and 150 μM, respectively). The figure presents two independent experiments, with each condition analyzed in triplicate. Graphs show PI/Annexin V–FITC overlay results for each condition, comparing data obtained using the modified PI staining protocol with those obtained using the classical protocol.

might still be considered acceptable in untreated cells, it is more than twice the value obtained with the modified protocol.

To investigate whether this result was dependent on the total concentration of PI used for the staining, we treated cells with a range of PI concentrations (2, 4, 6, 8, 16 μg of total PI in 1.5 mL of medium per well; Fig. 2B). No toxicity was observed, even at extremely high PI concentrations. For both methods, no particular variations in the necrotic cell counts were detected by increasing the concentration of PI used.

At all PI concentrations tested, the modified protocol, adding PI directly to the culture medium before detachment, consistently yielded a low necrotic fraction (Fig. 2B, upper panels). The same samples were then processed using the traditional workflow (centrifugation, washing, and PI staining), creating paired conditions for each experiment. This allowed us to compare each treatment both with minimal handling and after the standard manipulation steps. Under these last conditions, the number of PI-positive (necrotic) events increased markedly (Fig. 2B, lower panels), consistent with the effect shown in Fig. 2 A.

As shown in Fig. 2 C, performing a paired t-Test between the non-manipulated and manipulated cells, we observed a significant increase of damaged/dead cells associated with the centrifugation step (Paired t-Test: Live cells: Mean of differences −15.75, SD of differences 2.857, SEM of differences 1.166, 95% confidence interval −18.75 to −12.75; Dead cells: Mean of differences 15.59, SD of differences 3.133, SEM of differences 1.279, 95% confidence interval 12.31–18.88; both p-value <0.0001). Even in these paired experiments on relatively manipulation-resistant cells, the shape of the “healthy” population differed between

protocols. With PI added before detachment (top panels), the distribution was broader and showed a higher density of events in the central region, where viable cells are located. When PI was added after handling (bottom panels), the distribution appeared narrower, with fewer events in the viable-cell region.

In Fig. 3 A, we show the intensities of PI-stained samples from the previous experiments. In this case, by evaluating the fluorescent cells, the difference already suspected by considering the cell populations became evident. Even at extreme PI concentrations, no toxicity was observed. With the modified protocol, we detected less than 2% of necrotic cells, compared with the approximately 30% observed when PI was added after centrifugation (Fig. 3 A, lower graphs). These latter results were comparable to those obtained with the classic protocol (Fig. 3B). When data obtained across the different PI concentrations were pooled for analysis (Fig. 3 C), we show a significant difference between sample treated with our approach (modified) and samples decorated with PI after manipulation (Paired t-Test: Mean of differences 27.18, SD of differences 2.167, SEM of differences 0.9689, 95% confidence interval 24.49–29.87; p-value <0.001).

Based on these results, we tested the modified protocol using 4 and 8 μg total PI in more manipulation-sensitive ("fragile") cell lines: H295R adrenocortical carcinoma cells (Fig. 4 A) and HEK-293T embryonic kidney cells (Fig. 4B). As shown in the upper panel of both Fig. 4A-B, the centrifugation/manipulation step caused an increase in the number of dead cells relative to the live ones. This manipulation step had a major impact on the percentage of live cells in H295R and HEK-293T strains compared with the SK-LU−1 cell line. This is evident from the flattening of the cell populations in FACS analysis. In the lower panel of both Fig. 4 A and 4B, we show the intensity and percentage of PI-stained cells with histograms. For H295R, we accounted for about 15–22% necrotic cells with the modified protocol; the same samples, stained with PI only after a centrifugation step, rose up to about 75% of PI-positive cells. Similar results were obtained for HEK-293T, with necrotic cells below 4% using our modified protocol that increased to about 50% with PI addition after centrifugation. Moreover, necrotic-cell levels measured with the classical protocol did not match what we observed on the plate. At such high "necrosis" rates, we would expect visibly stressed, detaching, non-viable cells; however, this was not consistent with the morphology seen by light microscopy (data not shown). To optimize the PI dose for robust necrosis detection, we treated all three cell lines overnight with 50% DMSO to generate a high-death condition. This treatment produced ≥ 90% dead cells, as also indicated by the classical protocol (Fig. 5, right blue panels), and was used to benchmark PI amounts. As shown in Fig. 5, across all tested PI doses (2, 4, 6, 8, and 16 μg total PI) and in all three cell lines (SK-LU−1, HEK-293T, and H295R), the fraction of dead cells remained consistently ≥ 90% and did not increase with higher PI. The main difference was the PI fluorescence intensity, which rose with PI dose, while dead-cell identification remained unchanged.

To validate the proposed modification, we compared our methodology with the commercial LIVE/DEAD™ Cell Imaging Kit, a well-established two-color assay for cell viability quantification. In particular, the live cell component produces an intense, uniform green fluorescence in live cells, while the dead cell component produces a predominantly nuclear red fluorescence in cells with compromised cell membranes. For each cell line, untreated cells were stained and viable cells were quantified as described in the Materials and Methods section. As shown in Fig. 6, the bar plot on the left illustrates the comparison of cell viability obtained using our method and the LIVE/DEAD™ Cell Imaging Kit. Across all analyzed cell lines, no significant differences were observed between the two methods (SK-LU−1: mean modified: 93%, mean kit: 90.75%; HEK-293T: mean modified: 97.92%, mean kit: 97.08%; H295R: mean modified: 91.07%, mean kit: 86.90%. t-Test p-value not significant between modified and kit method: p-value 0.6238, 0.4916 and 0.1969 respectively), with comparable and overlapping percentages of viable cells. These quantitative results were consistent with the microscopy images shown in Fig. 6. In both the proposed modified method and the commercial kit, PI staining was detected exclusively in round-shaped cells that had lost plasma membrane integrity, while cells with normal morphology remained unstained.

To assess whether the proposed modified method is influenced by centrifugation or centrifugation speed, untreated H295R cells were stained with PI prior to any harvesting step. PI-positive cells were quantified at three sequential stages: before centrifugation, after centrifugation at different g-forces (100, 300, or 500 g), and after PI addition following the classical post-harvest protocol (n = 6 for each experimental group). As shown in Fig. 7A, when PI staining was performed before any handling, the fraction of PI-positive cells did not change significantly after centrifugation at any of the tested speeds. In contrast, a significant increase in PI-positive cells was detected when PI was added after centrifugation, independently of centrifugation speed (100 g: Mean of differences: 19.00, SD of differences: 11.01, SEM of differences: 4.494, 95% confidence interval: 7.447–30.55; 300 g: Mean of differences: 19.67, SD of differences: 7.967, SEM of differences: 3.252, 95% confidence interval: 11.31–28.03; 500 g: Mean of differences: 24.33, SD of differences: 2.944, SEM of differences: 1.202, 95% confidence interval: 21.24–27.42; all paired t-Test p-value <0.01). Consistently, Fig. 7B shows that PI-positive fractions were similar across centrifugation speeds with both staining approaches. Notably, when PI was added after centrifugation, these cells showed the same PI-positive bias even at the lowest speed, comparable to that observed at higher speeds. In the same experiment, we also measured the absorbance of the supernatant, which reflects the fraction of cells that were not pelleted by centrifugation. As shown in Fig. 7C, absorbance decreased with increasing centrifugation speed, indicating for H295R a greater cell loss at 100 g than at 500 g. This is also confirmed by the cell counts in subsequent steps, which, as expected, have the opposite pattern (Supplementary Figure.1). Taken together, these results demonstrate that the modified protocol is independent of centrifugation speed, whereas post-harvest PI addition contributes to artifactual increases in PI positivity and can influence overall cell recovery.

As a final analysis, we attempted to use our method with a commercial apoptosis kit. Again, the 4 μL of PI used to assess necrotic cells were added 30 min before detaching the cells and then again after centrifugation, according to the manual. Fig. 8 summarizes the results of the two experiments where SK-LU−1 and H295R were treated 48 h with cisplatinum (CDDP) to induce apoptosis. To keep the figure readable, we show the overlaid FACS plots from three independent wells per condition (±CDDP). Experiments were performed in biological replicate for both cell lines, and all plots, including gates and populations, are provided in Supplementary Figure 2. While CDDP effects were detectable with both protocols, the main difference concerns quantification of necrotic/late-apoptotic cells. Our modified method captured CDDP-induced damage, but consistently reported fewer dead cells than the classical protocol. This bias was also evident when observing all PI-positive cells (Necrotic: SK-LU−1: Mean of differences: 10.72, SD of differences: 11.62, SEM of differences: 3.354, 95% confidence interval: 3.342–18.11; H295R: Mean of differences: 5.679, SD of differences: 3.699, SEM of differences: 1.068, 95% confidence interval: 3.328–8.030, both p-value <<0.01; Late Apoptotic: SK-LU−1: Mean of differences: 8.128, SD of differences: 12.60, SEM of differences: 3.638, 95% confidence interval: 0.1212–16.13, p-value: 0.0472; H295R: Mean of differences: 35.77, SD of differences: 3.911, SEM of differences: 1.129, 95% confidence interval: 33.28–38.25, p-value <<0.01). Even in this experiment, when necrosis was induced by the treatment our modified method was able to identify it with comparable accuracy to the classical one. The results were consistent with those observed in previous experiments using PI alone.

Discussion

Assessing programmed cell death is central to oncology research and

early drug screening. Among the many available assays [20], the Annexin V/PI FACS method remains the most widely used reference for distinguishing apoptotic (Annexin V–positive) from necrotic (PI-positive) cells [17]. Despite its versatility, it can overestimate necrosis in primary cells and in specific cell lines [18], a limitation that has prompted multiple modifications, often at the cost of the method's simplicity and practicality [18,21–23].

Here we propose a minimal change that preserves the original workflow and requires no reagents beyond those already included in standard commercial kits. The key modification is to expose cells to PI directly in the culture medium before detachment, so that PI is taken up only by cells with genuinely compromised membranes. PI is then washed out prior to harvesting, preventing staining of cells that become permeabilized during post-harvest steps (e.g., detachment, centrifugation, resuspension). Within our experimental timeframes and on ice, we did not observe delayed staining effects. While previous work suggested that PI-related artifacts may arise from RNA binding and proposed RNase treatment [18], our data indicate that a major source of PI-positive events is handling itself, with an impact that strongly depends on the cell line.

Across epithelial models with different susceptibility to manipulation, this simple modification consistently reduced handling-associated overestimation of necrotic/late-apoptotic populations. Although our analysis focuses on the technical variability between the classical protocol and our modified method, we also observe the same underlying pattern at the biological level, with the magnitude of the effect shaped by the specific characteristics of each cell line used. The effect was most pronounced in "sensitive" lines such as H295R and HEK-293T, but it was also evident in SK-LU−1, where the classical protocol reported roughly twofold higher necrotic fractions than the modified approach. Importantly, the modified readout agreed with an independent commercial LIVE/DEAD assay, supporting its accuracy for necrotic-cell quantification.

Although FACS remains essential for sensitive multiparametric analysis, necrotic-cell counts obtained with our approach were comparable even when using a standard fluorescent cell counter, suggesting that gating is not the primary driver of the necrosis discrepancy. We therefore minimized filtering to reduce potential selection effects on morphologically atypical apoptotic cells, while noting that future users can readily apply their instrument- and cell-specific settings.

We also evaluated centrifugation as a potential mitigation strategy. While optimizing speed can reduce artifacts in some models, in fragile lines (e.g., H295R) lowering speed can compromise pellet recovery and still does not resolve the PI-positive bias. In contrast, our approach was effectively centrifugation-independent because staining occurs under physiological conditions before any handling, and PI is removed before subsequent manipulations. This rationale extends to common steps for adherent cells, including detachment methods, washes, and resuspensions.

Finally, the modification is designed to improve necrotic-cell accuracy without compromising apoptosis assessment. We optimized PI conditions using controlled membrane damage (DMSO) and confirmed that cisplatin-induced apoptosis was detectable in two cell lines with both protocols; the main divergence remained the inflated necrotic/late-apoptotic fraction produced by the classical workflow, including in untreated controls. This supports the view that the core issue is not the drug or its apoptotic potential, but the handling sensitivity of certain models. Reducing necrotic false positives can help avoid misinterpreting treatment effects and may reduce the risk of deprioritizing promising compounds during early screening. Overall, our modification preserves the main strengths of the Annexin V/PI assay over indirect enzymatic readouts: it directly quantifies apoptotic and necrotic cells and is less susceptible to interference from experimental compounds that can affect enzyme-based assays, while also improving the robustness of necrotic-cell measurement. Furthermore, the modification is rapid, cost-neutral, and straightforward to implement, since PI is already included in most commercial kits and no additional reagents are required.

Abbreviations

CDDP Cisplatinum
min minutes
PCD Programmed Cell Death
PI Propidium Iodide
PS Phosphatidyl-Serine
PBS Phosphate Buffered Saline
FBS Fetal Bovine Serum

CRediT authorship contribution statement

Laura Saba: Writing – original draft, Methodology, Investigation. **Carlotta Evaristo:** Methodology. **Aurora Schiavon:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Marco Lo Iacono:** Writing – original draft, Supervision, Formal analysis, Conceptualization. **Massimo Terzolo:** Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Claudia Giachino:** Methodology. **Chiara Longo:** Methodology, Investigation. **Jessica Petiti:** Writing – original draft, Methodology, Investigation, Conceptualization.

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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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2026.08.004.

Data availability

All data are included in the manuscript.

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