Monitoring the progression of cell death and disassembly of dying cells by flow cytometry

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The use of annexin A5 and propidium iodide/7-aminoactinomycin D stains to measure cell death by flow cytometry has been considered the gold standard by most investigators. However, this widely used method often makes the assumption that there are only three types of particles in a sample, that is viable, apoptotic and necrotic cells. In order to study the progression of cell death in greater detail, in particular how apoptotic cells undergo fragmentation to generate membrane-bound vesicles known as apoptotic bodies, we have established a new flow cytometry-based protocol to accurately and rapidly measure the cell death process. This protocol utilises a combination of annexin A5 and TO-PRO-3 (a commercially available nucleic acid-binding dye that stains early apoptotic and necrotic cells differentially), and a logical seven-step analytical approach to distinguish six types of particles in a sample, including apoptotic bodies and cells at three different stages of cell death. The protocol requires 1-5 h for sample preparation (including induction of cell death), 20 min for staining and 5 min for data analysis.

Keywords: Apoptosis, Apoptotic bodies, Apoptotic cell disassembly, Cell death, Cell viability, Flow cytometry, Necrosis

INTRODUCTION

Annexin A5 binding and propidium iodide/7-aminoactinomycin D uptake assay

Determining cell viability and monitoring the progression of cell death is a key aspect of many fields of biological and medical research, and a routine procedure in many laboratories. For experimental conditions where cells from *in vitro*, *ex vivo* or *in vivo* origins can be maintained in single cell suspension, levels of dying/dead cells are often determined by the flow cytometry-based annexin A5 (A5) protein binding and propidium iodide (PI) dye uptake assay[1-8]. The assay is based on the ability of cells undergoing apoptosis (a form of programmed cell death) to expose phosphatidylserine (PS) on the cell surface following caspase activation, and subsequently allowing the binding of fluorochrome-conjugated A5 (e.g. A5-FITC) to surface PS in a calcium-dependent manner[9,10]. Since healthy viable cells don't usually expose a high levels of PS on the outer leaflet of the plasma membrane, the levels of A5 staining is used to distinguish viable cells from apoptotic cells. The use of A5 as the only parameter to monitor cell viability by flow cytometry can be used to determine whether cells are simply dead or alive[11,12]. In order to further differentiate early apoptotic cells from membrane permeabilised cells (e.g. late apoptotic cells/secondary necrotic cells and primary necrotic cells[13,14]), the membrane impermeable nucleic acid binding dye PI can be used in combination with A5, with PI only entering and staining membrane permeabilised cells[1-8]. Using this approach, the relative proportion of viable, early apoptotic and necrotic cells in a reasonably sized sample (e.g. 20,000 cells) can be determined rapidly (in less than 1 min) by most contemporary flow cytometers. It is worth noting that PI is often replaced by another membrane impermeable nucleic acid binding dye, 7-aminoactinomycin D (7-AAD), due to the longer wavelength emission of 7-AAD to allow better multiplexing with other dyes and fluorochromes (e.g. phycoerythrin)[15].

The resultant flow cytometry data is typically analysed using a two-step analytical approach, denoted here as the 'traditional' two-step gating strategy[16]. First, particles that are larger in size (forward scatter, FSC) and high in granularity (side scatter, SSC) are identified as cells (**Supplementary Fig. 1**). Second, cells are then subdivided into three populations based on A5 and 7-AAD staining, resulting in the classification of $A5^-$ $7\text{-}AAD^-$ as viable cells, $A5^+$ $7\text{-}AAD^-$ as early apoptotic cells, and $A5^+$ $7\text{-}AAD^+$ as necrotic cells (**Supplementary Fig. 1**).

Development of a new assay to better monitor the progression of cell death and apoptotic cell disassembly

Although the A5 binding and PI/7-AAD uptake assay and subsequent data analysis is very effective in determining cell viability[1-8], it was designed to identify only three

types of particles in a sample (i.e. viable, early apoptotic and necrotic cells) but not subcellular fragments generated from dying cells, in particular apoptotic bodies.

Apoptotic body is a type of extracellular vesicle that is typically 1-5 μm in diameter[14,17]. Apoptotic bodies are generated during apoptosis (a process known as apoptotic cell disassembly) and can be observed in many (but not all) cell types including T cells, monocytes, fibroblast, endothelial cells and epithelial cells[7,8,18-24]. The formation of apoptotic bodies is thought to play an important role in promoting the clearance of apoptotic cells by phagocytes[25,26]. Apoptotic bodies can also carry DNA, microRNA, proteins and lipids to mediate communication between cells[27-29]. Since the formation of apoptotic bodies is a key cellular process downstream of cell death, we have recently developed a new flow cytometry-based method that can accurately and rapidly measure the extent of apoptotic cell disassembly, as well as analysing the progression of cell death in greater detail[18,19]. This method utilises a combination of A5-FITC and the nucleic acid binding dye TO-PRO-3, together with flow cytometry data analysis using a new seven-step gating strategy (see Experimental Designs for details) to identify six types of particles in a sample. The principle of this approach in monitoring cell death and disassembly by flow cytometry is shown schematically in **Figure 1**. This method takes advantage of the recent discovery that pannexin 1 (PANX1) membrane channels are activated by caspase-mediated cleavage during early stages of apoptosis (prior to apoptotic morphology and PS exposure) and allow the entry of TO-PRO-3[18,30]. Thus, $A5^-$ early apoptotic cells can be identified based on TO-PRO-3 uptake via PANX1, with $A5^-$ early apoptotic cells exhibiting an approximately 9-fold increase in TO-PRO-3 staining compared to viable cells[18] (**Fig. 1 and Supplementary Fig. 2**). $A5^+$ early apoptotic cells are identified based on both A5-FITC binding and TO-PRO-3 staining. Strikingly, membrane permeabilised cells allow the entry of TO-PRO-3 independent of PANX1 activity, and resulted in an approximately 170-fold increase in TO-PRO-3 staining compared to viable cells[18] (**Fig. 1 and Supplementary Fig. 2**). The combination of A5-FITC binding and high levels of TO-PRO-3 staining facilitates the identification of necrotic cells without the need for PI or 7-AAD staining (**Fig. 1**). Utilising this newly described analytical approach, subcellular fragments like apoptotic bodies and $A5^-$ particles/debris can also be identified based on A5-FITC binding and relative size compared to whole cells[18,19] (**Fig. 1**).

Potential applications of the protocol

Analysing cell death by flow cytometry using the A5 binding and PI/7-AAD uptake assay and the traditional two-step gating method has been the gold standard in biological and medical research for many years[1-8]. However, it is evident that the new protocol as described above has a number of advantages over the A5 binding and

PI/7-AAD uptake assay. First, the new A5 binding and TO-PRO-3 uptake protocol can identify an additional stage of cell death (i.e. A5$^-$ early apoptotic cells) as well as two types of subcellular fragments in a sample, thus improving the resolution of the flow cytometry data. This is particularly relevant for the study of cell death pathways and the apoptotic cell disassembly process. It is also worth noting that this assay could potentially be used to identify necroptotic cells that are poor in A5 binding[31], possibly exhibiting a A5$^-$ and TO-PRO-3 high characteristics. Second, the availability of A5 conjugated to a variety of fluorochromes and dyes that are similar to TO-PRO-3 with different excitation/emission profiles commercially will provide investigators the flexibility to design a combination that is most suitable for their experiments. It is worth noting that the protocol can be easily scaled down for high throughput screening or scaled up for large sample volume.

Limitations

The protocols is dependent on two key events that occurs during apoptosis, that is the activation of PANX1 by caspases at an early stage of apoptosis, and the subsequent exposure of PS prior to membrane lysis. Although PANX1 channels are thought to be expressed by many cell types[32-34], the levels of PANX1 at the plasma membrane may differ between cell types. Thus, the levels of TO-PRO-3 staining by early apoptotic cells can also vary accordingly. Likewise, whether early apoptotic cells will uptake TO-PRO-3 prior to PS exposure may also differ depending on the cell type. Nevertheless, this limitation will only affect the detection of A5$^-$ early apoptotic cells. Besides PANX1 activity, the levels of PS exposure during apoptosis can also vary depending on the cell type[35,36]. Therefore, in instances where PANX1 activity and PS exposure is low in certain apoptotic cell type, this protocol (or the previous A5 binding and PI/7-AAD uptake assay) would not be suitable to identify early apoptotic cells. It is worth noting that the ability of different flow cytometers to detect small particles also differs greatly, with only certain flow cytometers capable to accurately detect particles at the submicron range[37,38]. Although apoptotic bodies are generally considered to be 1-5 μm in diameter[14,17] and can be detected by most contemporary flow cytometers, it is important to acknowledge that apoptotic bodies at approximately 1 μm or smaller may not be measured accurately and could underestimate the apoptotic cell disassembly process. Nevertheless, if a flow cytometer is available to detect particles at the submicron range, smaller apoptotic bodies as well as microparticles (typically 0.1-1 μm) can potentially be measured (may require switching the FSC parameter to Log scale and the inclusion of sizing beads).

Experimental Design

The protocol can be divided into three key stages:

1. Preparation of samples for cell death induction
2. Preparation of stains and sample staining
3. Data acquisition by flow cytometry and data analysis

Preparation of samples for cell death induction

In this protocol, we will describe the induction of human Jurkat T cells (a non-adherent cell line) to undergo apoptosis by anti-Fas[18,19] or undergo primary necrosis by the membrane lytic peptide NaD1[39,40]. Similar procedures can be adapted to other non-adherent cell lines (e.g. human THP-1 monocytic cells and mouse EL4 T cells) or primary cells (e.g. human peripheral blood monocytes and mouse thymocytes), and induced to undergo cell death by other stimulus such as ultraviolet irradiation, dexamethasone, staurosporine and hyperthermic conditions[18,19,41,42]. It is worth noting that immediately after cell death induction, cells are transferred to test tubes (e.g. 5 ml round-bottom tubes) that can be used directly for sample staining and sample acquisition by flow cytometry. This is particularly relevant for the analysis of apoptotic cell disassembly to minimize sample handling procedures like pipetting and centrifugation steps. Such sample handling procedures could potentially affect the levels of apoptotic body formation. For adherent cells, it is inevitable that some form of sample handling is necessary (e.g. trypsin and/or EDTA treatment) to dissociate cells from the cell culture surface, and subsequently combining with dying cells that are detached into the culture supernatant for flow cytometry analysis. Although this protocol (like A5 binding and PI/7-AAD uptake assay) can be used to monitor adherent cells undergoing cell death and disassembly, we recommend the use of additional cell death assays (e.g. microscopy analysis, caspase activity assay and lactate dehydrogenase release assay) in combination with this protocol to make the most appropriate conclusion.

Preparation of stains and sample staining

Although this protocol focuses on staining samples with A5-FITC and TO-PRO-3, the protocol can also be adapted to include additional antibody-based staining and cell-counting beads. For example, anti-CD4-PE-Cy7 and anti-CD8a-PE can be included in the staining master mix in combination with A5-FITC and TO-PRO-3 to stain primary mouse thymocytes[18]. In instances where additional stains are used, it is important to ensure appropriate single stain controls are prepared to perform fluorescence compensation. Furthermore, if A5-FITC cannot be used due to conflicts with cell stains (e.g. carboxyfluorescein succinimidyl ester) or the expression of green fluorescent protein, other fluorochrome-conjugated A5 such as A5-PE can be used.

Besides flow cytometry analysis as described below, cells and cell fragments stained using this protocol can also be examined by live cell fluorescence microscopy techniques, as well as analysed using an imaging flow cytometer[18,19].

Furthermore, in situations where fluorochrome-conjugated A5 cannot be used, monitoring TO-PRO-3 uptake alone can differentiate viable cells, early apoptotic cells (including both $A5^-$ and $A5^+$ populations) and necrotic cells using an alternative gating strategy (**Supplementary Fig. 3**). It should be noted that similar analysis of different dying cell populations with 7-AAD staining alone have been established in previous studies[43]. Although this alternative staining and gating approach is sufficient to determine cell viability, information regarding the apoptotic cell disassembly process is reduced due to the lack of A5 parameter to separate apoptotic bodies from $A5^-$ particles/debris (**Supplementary Fig. 3**).

Data acquisition by flow cytometry and data analysis

To ensure accurate identification of all six types of particles (cells and cell fragments) in a sample, it is critical during data acquisition that all parameters (i.e. FSC, SSC, A5-FITC and TO-PRO-3) are adjusted to the optimal settings. Example flow cytometry plots of FSC versus SSC and A5-FITC versus TO-PRO-3 for control cells and cells undergoing apoptosis is shown in **Figure 2**. Adjusting appropriate settings (i.e. the FSC photodiode amplifier gain and photomultiplier tube settings for SSC, A5-FITC and TO-PRO-3 channels) in the data acquisition program to generate flow cytometry plots that are comparable to **Figure 2** is necessary to perform the subsequent data analysis.

The new seven-step gating strategy (denoted as STEP 1 to STEP 7) is shown in **Figure 3a**. The logic underpinning this electronic gating strategy is based on the principle as described in **Figure 1**. Although this new gating scheme seems more complex than the traditional two-step method (**Supplementary Fig. 1**), it is logical, clear and can be performed within 5 min to identify six types of particles in a sample (**Fig. 3a and b**). It is worth noting that in our previous studies[18,19], we have also used the 7-AAD parameter to identify necrotic cells and stained the sample with a combination of A5-FITC, TO-PRO-3 and 7-AAD. To accommodate 7-AAD staining in the data analysis when all three viability stains are used simultaneously, STEP 1 of the seven-step gating strategy is replaced with an ALTERNATIVE STEP 1, with 7-AAD plotted against A5-FITC to identified A5-FITC high and 7-AAD high particles as membrane permeabilised cells (**Fig. 3c**).

Besides the identification of cells at different stages of cell death, the new protocol can also be used to quantify the extent of apoptotic cell disassembly based on the relative levels of apoptotic bodies and $A5^+$ early apoptotic cells[18,19]. The apoptotic cell disassembly process can be measured by the apoptotic body formation index

calculated from the number of apoptotic bodies divided by the number of A5$^+$ early apoptotic cells[18,19] (data obtained from STEP 7 of the new gating strategy). Monitoring the disassembly of apoptotic T cells, thymocytes, fibroblast and monocytes using this approach has been applied in our recent studies[18,19]. It is worth noting that since cell-counting beads can be included in the staining procedure (as described above), the absolute number of apoptotic bodies in a sample can also be determined. Nevertheless, caution must be taken when analysing the formation of apoptotic bodies, as the levels of apoptosis and necrosis in the sample must be taken into consideration[18,19]. Thus, the ability of this protocol to determine the levels of viable cells, cells at three different stages of cell death and apoptotic bodies simultaneously is a powerful method to monitor the apoptotic cell disassembly process.

MATERIALS

REAGENTS

- Cultured human Jurkat T cell line (clone E6-1, ATCC, cat. no. TIB-152) Δ **CRITICAL** Use cells that are mycoplasma free, as mycoplasma contamination can affect the levels of TO-PRO-3 staining.
- RPMI 1640 medium (Life Technologies, cat. no. 22400-089)
- Penicillin-streptomycin mixture (Life Technologies, cat. no. 15140122)
- MycoZap (Lonza, cat. no. VZA-2012)
- Fetal calf serum (FCS; Gibco, cat. no. 10099-141)
- Bovine serum albumin (BSA; Sigma, cat. no. A7030)
- Anti-Fas, Hu activating (clone CH11, Millipore, cat. no. 2397046)
- Recombinant plant defensin NaD1 (rNaD1; generated according to Lay *et al.* 2012[44])
- A5-FITC (BD Biosciences, cat. no. 556419)
- 10× A5 Binding Buffer (BD Biosciences; cat. no. 556454)
- TO-PRO-3 iodide (Life Technologies, cat. no. T3605) **! CAUTION** TO-PRO-3 may cause skin, eye and respiratory irritation. Avoid direct contact, use gloves while preparing and using TO-PRO-3.
- 7-AAD (Life technologies, cat. no. a1310) **! CAUTION** 7-AAD is carcinogenic and has reproductive toxicity. Avoid direct contact, use gloves while preparing and using 7-AAD.

EQUIPMENT

- Tissue culture equipment

- 15 ml centrifuge tubes (Cellstar, cat. no. 188271)
- 1.5 ml microcentrifuge tubes (Sarstedt, cat. no. 72.690.001)
- 5 ml Polystyrene round bottom test tubes (Falcon, cat. no. 352008)
- 4^{O}C and -20^{O}C refrigerators
- Tissue culture incubator (37^{O}C, 5% CO_2; vol/vol)
- Centrifuge
- Flow cytometer (FACSCanto II equipped with 15 mW Argon-Ion (488 nm) and 12 mW red diode (633 nm) lasers, 530/30 and 660/20 bandpass filters were used for FITC and TO-PRO-3 signals, respectively; BD Bioscience)
- FACSDiva 6.1.1 software (BD Bioscience)
- FlowJo 8.8.6 software (Tree Star Inc.)

REAGENT SETUP

- **Culture medium** Supplement RPMI 1640 medium with 10% (vol/vol) FCS, 0.2% (vol/vol) MycoZap, 50 IU ml^{-1} penicillin and 50 µg ml^{-1} streptomycin.
- **Incubation buffer** Supplement RPMI 1640 medium with 1% (wt/vol) BSA.
- **A5-FITC and TO-PRO-3 staining master mix** Dilute A5-FITC 1 in 100 and TO-PRO-3 to 1 µM in 2× A5 Binding Buffer. Δ **CRITICAL** The staining master mix should be made fresh for no more than 30 min prior to the cell staining step and kept in the dark at RT (22^{O}C). Since fluorochrome-conjugated A5 are often purchased as 'test', the investigator should perform a titration of A5 binding to apoptotic cells to determine the optimal amount of A5 is required for the assay.

OPTIONAL Include 7-AAD (2 µg ml^{-1}) in the staining master mix.

PROCEDURE

1. Culture human Jurkat T cells in culture medium at 37^{O}C with 5% (vol/vol) CO_2.

2. Determine cell number in culture. Pellet the necessary amount of cultured cells at 300 *g* at RT for 5 min, discard supernatant and resuspend cells at 4×10^6 cells ml^{-1} in incubation buffer. Ensure the incubation buffer is warmed up to 37^{O}C prior to use.

Δ **CRITICAL STEP** A small proportion of cells in culture could spontaneously undergo apoptosis and apoptotic cell disassembly, and subsequently becoming necrotic. To eliminate these dead cells and cell fragments prior to sample preparation, we recommend pelleting the cultured cells at 30 *g* at RT for 10 min, discard supernatant (containing dead cells and cell fragments) and resuspend cells for cell counting.

3. Aliquot 50 µl of cell suspension (i.e. containing 2×10^5 cells) per sample into individual test tubes.

Δ **CRITICAL STEP** It is important to include additional samples for unstain and single stain controls.

4. Prepare a 2× treatment mixture in incubation buffer to induce cell death. Follow the method for induction of apoptosis (option A) and necrosis (option B).

(A) Induction of apoptosis by anti-Fas (via Fas activation)

(i) Prepare anti-Fas at 62.5 ng ml^{-1} in incubation buffer and add 50 µl to test tubes containing 50 µl of cell suspension. For vehicle control (i.e. cells not induced to undergo cell death), prepare a mixture containing the same volume of compound solvent in incubation buffer and add 50 µl to test tubes containing 50 µl of cell suspension.

(ii) Incubate at 37^OC with 5% (vol/vol) CO_2 for 4 h to induce apoptosis.

(B) Induction of necrosis by the plant defensin rNaD1 (via direct plasma membrane permeabilisation)

(i) Prepare rNaD1 at 20 µM in incubation buffer and add 50 µl to test tubes containing 50 µl of cell suspension. Prepare vehicle control as per (A).

(ii) Incubate at 37^OC with 5% (vol/vol) CO_2 for 30 min to induce necrosis.

Δ **CRITICAL STEP** Mix well and avoid samples being placed on the side of the test tube.

OPTIONAL Other compounds can be used in this step to induce cell death or modulate the cell death process. The relative volume of cell suspension to treatment mixture can also be changed (e.g. 80 µl of cell suspension mixed with 20 µl of 5× treatment mixture). If evaporation of samples is a potential concern when using such small sample volume, the volume of samples and staining master mix can be increased by 2 fold.

5. After induction of cell death, immediately add 100 µl of A5-FITC and TO-PRO-3 staining master mix to each sample and incubate at RT for 10 min in the dark. Mix well by gently tapping the test tube. Δ **CRITICAL STEP** It is important to perform this step at RT, for at least 10 min but less than 20 min.

? TROUBLESHOOTING

6. Place samples on ice and in the dark. Δ **CRITICAL STEP** It is preferable to analyse samples by flow cytometry as soon as possible. However, the samples can be kept on ice for 1-2 h. Do not fix samples.

7. Analyse cells and cell fragments by flow cytometry. Set FSC, SSC, A5-FITC and TO-PRO-3 parameters according to **Figure 2**. Collect at least 20,000 events. Δ **CRITICAL STEP** TO-PRO-3 staining for early apoptotic cells will gradually increase over time. Thus, it is preferable to perform data acquisition within 1 h. **? TROUBLESHOOTING**

8. Data analysis using the FlowJo program to perform electronic gating (see **Figure 3**).

- **TIMING**

Sample preparation for the induction of cell death can be completed in less than 1 h.
Induction of apoptosis and necrosis in cell lines requires 0.5-4 h.
Data acquisition by flow cytometry and data analysis requires 1-1.5 h.

? TROUBLESHOOTING

Table 1 Troubleshooting table

Problem	**Possible reason**	**Possible solution**
Flow cytometry profile does not match examples in Figure 2	Settings on the flow cytometer are not optimal	Adjust the FSC photodiode amplifier gain and photomultiplier tube settings for SSC, A5-FITC and TO-PRO-3 channels accordingly
	Cell type of interest don't increase the cell surface levels of PS and/or don't have a sufficient amount of caspase-activated PANX1 channels at the plasma membrane during apoptosis	See below for Low A5 and Low TO-PRO-3 signal compared to viable cell controls
Low A5 signal compared to viable cells control	Staining step was performed at a lower temperature (e.g. on ice) and for less than 10 min	Make sure the staining step is performed at RT (~20^{O}C) and for at least 10 min
	Bleaching of fluorochrome-conjugated A5 prior to data	Incubate fluorochrome-conjugated A5 in the dark to avoid exposure of

	acquisition Cell type of interest don't increase the cell surface levels of PS during apoptosis Cell type of interest may not be undergoing apoptosis	reagents and samples to light Use TO-PRO-3 uptake to distinguish cells at different stages of cell death Use other methods (e.g. staining for cleaved caspases[45], TUNEL assay[46]) to examine whether apoptosis occurs in the sample
Low TO-PRO-3 signal compared to viable cell control	Staining master mix was prepared for hours in advance Cell type of interest don't have a sufficient amount of caspase-activated PANX1 channels at the plasma membrane Cell type of interest may not be undergoing apoptosis	Prepare staining master mix fresh immediately prior to cell staining Necrotic cells should still be identified by TO-PRO-3 staining, but $A5^-$ early apoptotic cells will not be separated from viable cells Use other methods to examine whether apoptosis occurs in the sample
High TO-PRO-3 signal in viable cell control	Staining step was performed for an extended period of time, leading to the uptake of TO-PRO-3 by viable cells.	Make sure the staining step is performed at RT (~20^OC) for at least 10 min but no longer than 20 min

ANTICIPATED RESULTS

In this protocol, we used the human Jurkat T cells and anti-Fas induced apoptosis as a model system to exemplify the new approach to analyse apoptotic cell death. When we compared this protocol (A5-FITC/TO-PRO-3 staining and new gating strategy) with the 3 stains approach (A5-FITC/7-AAD/TO-PRO-3 staining and new gating strategy) and the traditional 2 stains approach (A5-FITC/7-AAD staining and traditional gating strategy), the levels of viable, $A5^+$ early apoptotic and necrotic cells were comparable (**Fig. 4**). However, the levels $A5^-$ early apoptotic, apoptotic bodies and $A5^-$ particles/debris cannot be determined by the traditional 2 stains approach (**Fig. 4**). Good correlation was observed between this protocol (i.e. A5-FITC/TO-PRO-3 staining and new gating strategy) and the 3 stains approach (**Fig. 4**), demonstrating 7-AAD staining is not required to distinguish necrotic cells from early apoptotic cells. Besides Jurkat T cells, this protocol can be used to identify cells at

different stages of cell death and cell fragments generated from human THP-1 and U937 monocytic cells, primary human peripheral blood monocytes, human A431 squamous carcinoma cells, human umbilical vein endothelial cells, mouse thymocytes, mouse EL4 T cells and mouse embryonic fibroblast (data not shown).

When Jurkat T cells were induced to undergo primary necrosis by the plant defensin rNaD1, all three staining methods were effective in identifying the generation of necrotic cells (**Fig. 4**). It is worth noting that although membrane impermeable nucleic acid binding dyes like PI, 7-AAD and TO-PRO-3 alone are sufficient to monitor the generation of membrane permeabilised cells and a procedure used by many investigators to study primary necrosis, necroptosis and pyroptosis[39,40,47-52], we suggest it is equally important to demonstrate the lack of early apoptotic cells under experimental conditions that induces membrane lysis (**Fig. 4**). Interestingly, using the new gating strategy irrespective of staining methods, a relatively large amount of subcellular $A5^+$ particles (resembling apoptotic bodies) and $A5^-$ particles/debris were identified when cells were induced to undergo primary necrosis by rNaD1 (**Fig. 4**), indicating subcellular $A5^+$ and $A5^-$ particles/debris are released by permeabilised cells.

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AUTHOR CONTRIBUTIONS L.J., R.T. and I.K.H.P designed, performed and analysed most of the experiments with help and input from S.C., G.K.A. and S.P. A.A.B., M.D.H., L.J. and I.K.H.P. designed and carried out experiments with rNaD1. I.K.H.P., L.J. and R.T. wrote the manuscript with input from co-authors.

COMPETING INTERESTS STATEMENTS The authors declare no competing financial interests.

FIGURE LEGENDS

Figure 1. Schematic of annexin A5-FITC (A5-FITC) binding and TO-PRO-3 uptake by cells and cell fragments. The expected levels of A5-FITC and TO-PRO-3 staining, as well as the size (forward scatter, FSC) and granularity (side scatter, SSC) of cells and cell fragments is indicated on the right.

Figure 2. Flow cytometry plots of human Jurkat T cells for optimal data acquisition and analysis. Jurkat T cells were treated without or with anti-Fas to induce apoptosis.

Representative flow cytometry plots of FSC versus SSC and A5-FITC versus TO-PRO-3 are shown.

Figure 3. Electronic gating strategy for analysing cell death and apoptotic cell disassembly. **a**, Flow cytometry analysis showing the new seven-step electronic gating strategy used to identify viable cells, $A5^-$ early apoptotic cells, $A5^+$ early apoptotic cells, necrotic cells, apoptotic bodies and $A5^-$ particles/debris. **b**, Flow cytometry analysis displaying each type of cells and cell fragments gated according to **a** has distinctive levels of A5-FITC and TO-PRO-3 staining, as well as FSC and SSC properties. **c**, If the 7-AAD stain is also included in combination with A5-FITC and TO-PRO-3 to determine cell viability, STEP 1 of the new gating strategy can be replaced by an ALTERNATIVE STEP 1. Jurkat T cells were induced to undergo apoptosis by anti-Fas treatment in all indicated experiments.

Figure 4. Identifying cells at different stages of cell death and cell fragments based on different methods of staining and electronic gating strategy. Human Jurkat T cells were treated with anti-Fas (31.25 ng ml^{-1}, 4 h) to induce apoptosis or treated with the membrane lytic peptide rNaD1 (10 μM, 0.5 h) to induce necrosis (n = 3). The resultant data were analysed using three different staining approach and electronic gating strategy as indicated. Error bars represent s.e.m. Data are representative of at least two independent experiments.

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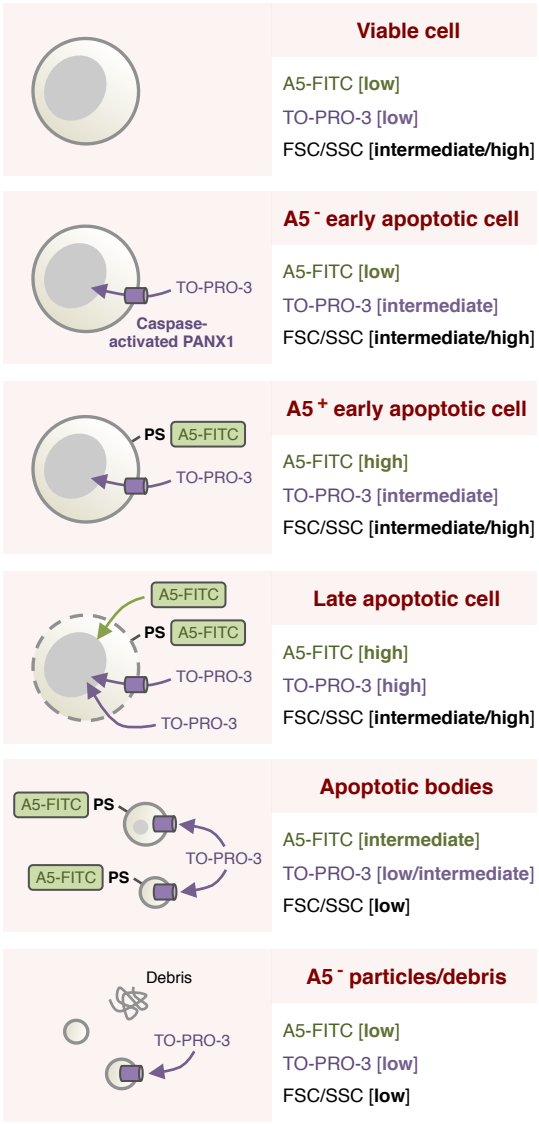

Figure 1. Schematic of annexin A5-FITC (A5-FITC) binding and TO-PRO-3 uptake by cells and cell fragments.

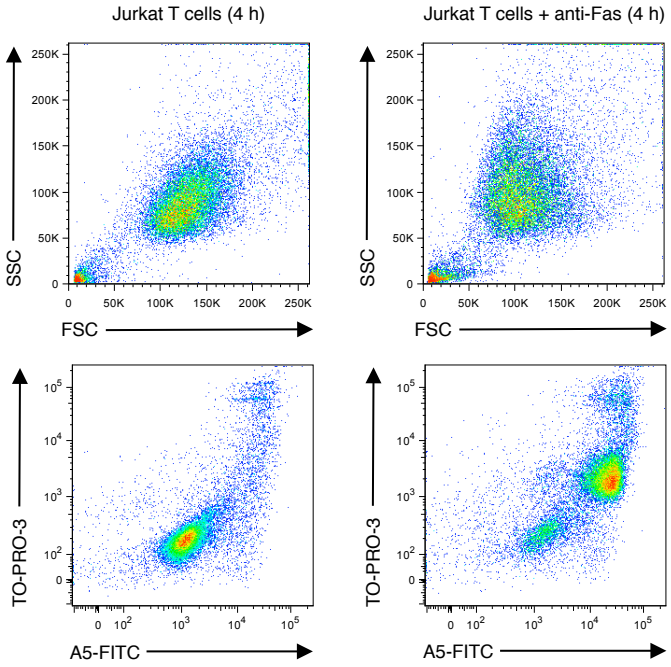

Figure 2. Flow cytometry plots of human Jurkat T cells for optimal data acquisition and analysis.

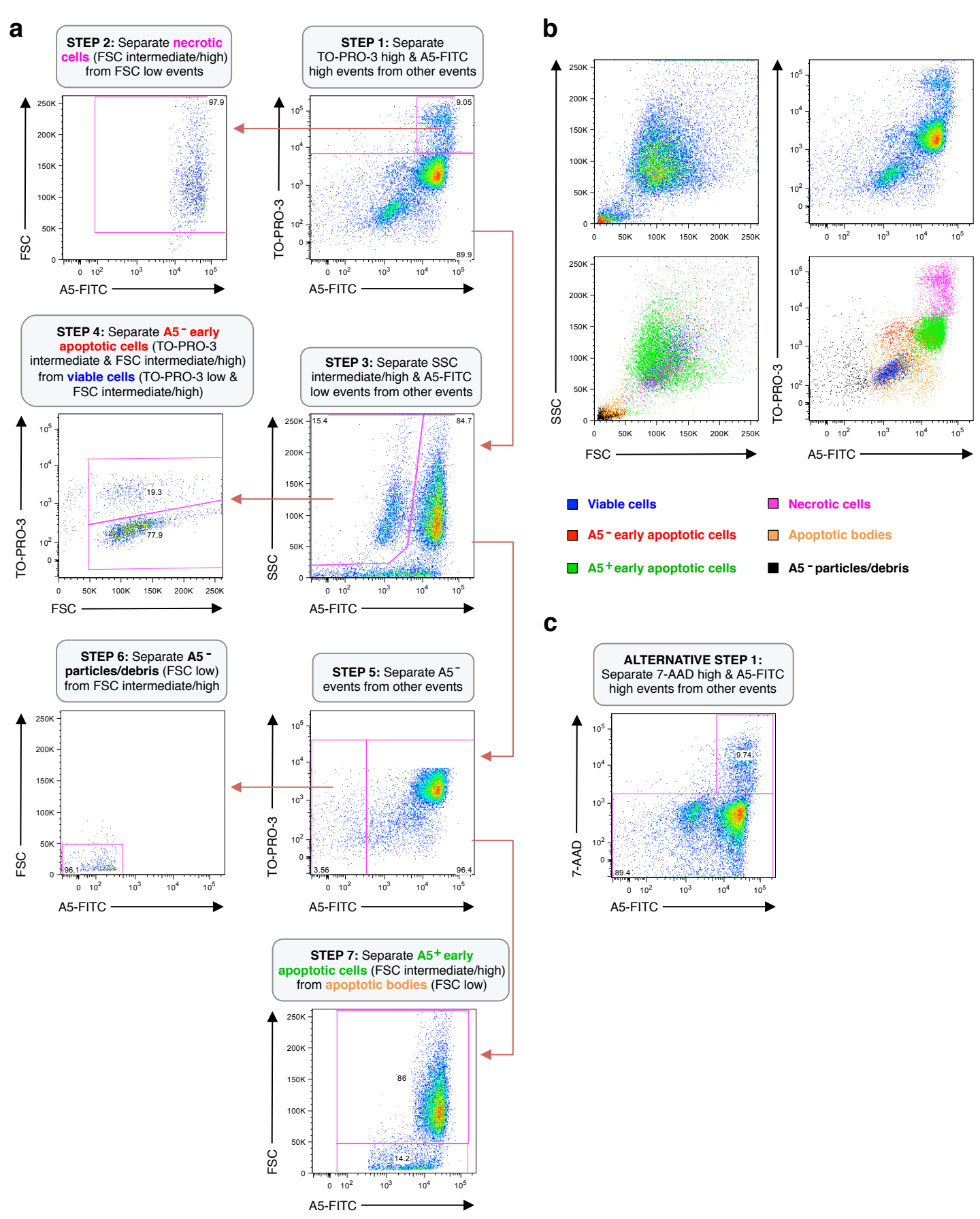

Figure 3. Electronic gating strategy for analysing cell death and apoptotic cell disassembly.

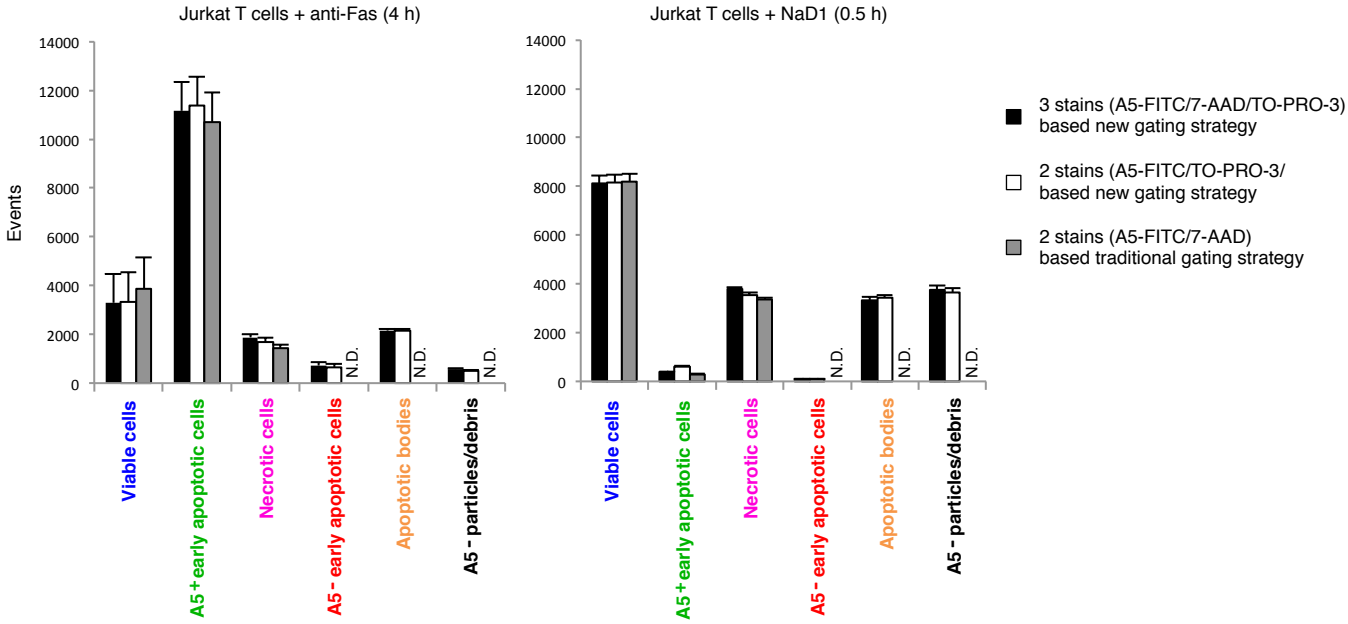

Figure 4. Identifying cells at different stages of cell death and cell fragments based on different methods of staining and electronic gating strategy.