Chapter 2

Flow Cytometry Enumeration of Apoptotic Cancer Cells by Apoptotic Rate

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Abstract

Most authors currently quantify the frequency of apoptotic cells in a given phenotypically defined population after calculating the apoptotic index (AI), i.e., the percentage of apoptotic cells displaying a specific linage antigen (LAg) within a population of cells that remain unfragmented and retain the expression of the LAg. However, this approach has two major limitations. Firstly, apoptotic cells fragment into apoptotic bodies that later disintegrate. Secondly, apoptotic cells frequently lose, partially or even completely, the cell surface expression of the LAg used for the identification of specific cell subsets. This chapter describes a flow cytometry method to calculate the apoptotic rate (AR) that takes into account both cell fragmentation and loss of lineage antigen expression on measurement of apoptosis using flow cytometry ratiometric cell enumeration that emerges as a more accurate method of measurement of the occurrence of apoptosis in normal and tumoral cell cultures.

Key words Apoptosis, Apoptotic rate, Apoptotic index, Cell enumeration, Accurate apoptosis measurement, Microbeads, Annexin V, Antigen loss, Cell fragmentation

1 Introduction

1.1 Apoptosis Measurement

The initial methods developed for the in vitro quantification of apoptosis measured phenomena associated with apoptosis in cultures at the population level, such as the assessment of nucleosomal DNA fragmentation after gel electrophoresis [1–3]. However, it soon became clear that individual cells undergo apoptosis in a heterogeneous and asynchronous manner [4]. It was therefore realized that the accurate measurement of apoptosis required methods that could identify apoptosis events at the single-cell level [5–11]. These methods revealed the heterogeneity of the apoptotic process to be correlated with cell phenotype—at least to a certain extent [12]. The ongoing development of flow cytometric techniques eventually made it possible to simultaneously identify and quantify apoptotic

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cells phenotypically defined by the expression of their surface lineage antigens (LAg).

The relevance of apoptosis has promoted active research into new methods of detecting these subcellular lesions at the single-cell level in complex cell mixtures both *ex vivo* and in cultured cells [5, 7, 13]. A good example is the use of annexin V for the detection of early apoptotic cells. In such cells, PS translocates from the inner side of the plasma membrane to the outer membrane leaflet, where it becomes exposed [14–16]. It can then be bound by annexin V, a phagocyte membrane protein [17]. The availability of fluorochrome-labeled recombinant soluble annexin V provides a useful tool for detecting and quantifying early apoptotic cells by flow cytometry [7, 8, 14]. The annexin V labeling method can be improved by the staining with the vital dye 7-amino-actinomycin D (7AAD) [11] to identify early and late apoptotic cells and necrotic ones. Cell washing, and choice of resuspension buffer, can affect the accuracy of measurements of apoptosis. It has been shown that wash cycles not only cause cell loss, but also affect the viability of cells, as well as the precision of repeat measurements. Therefore, wash cycles should be reduced to a minimum, which also reduces the time required for sample preparation.

1.2 Discrimination Between Whole Cells and Cell Fragments by Flow Cytometry

7AAD labeling can be used to discriminate between either viable and apoptotic whole cells, or cell fragments. Apoptosis led to the fragmentation of apoptotic cells into apoptotic bodies under different culture conditions (Fig. 1). Compared to whole cells, apoptotic bodies are smaller, consistent with the notion that one cell generates several apoptotic bodies. The inclusion of the latter in the cell analysis gate and their subsequent consideration as apoptotic cells result in an overestimation of the frequency of apoptosis and, therefore, discrimination between cells and apoptotic bodies is critical for accurate measurement of apoptosis.

As shown in Fig. 1, the discrimination between either viable and apoptotic whole cells or cell fragments was achieved by the analyses of both their bivariate profiles of size (FSC)/DNA staining with 7AAD (left panels), and their FSC/granularity (SSC) distribution (right panels). Contour plots in the top panels show that freshly purified $CD19^+$ lymphocytes formed a homogeneously sized population of viable cells that uniformly excluded 7AAD. After 24 h of culture (bottom panels) both apoptotic cells and apoptotic bodies emerged, but the remaining subset of viable B-cells maintain the characteristics from the original fresh B-lymphocytes, since they shared their FSC/SSC features (panel d), and did not take up 7AAD (contour levels under viable cell arrows in the panels c and d). In contrast, apoptotic cells showed a reduced FSC and a slightly increased SSC (panel d) that was coincident with variable 7AAD staining related to progression into late apoptosis (panel c). Finally, apoptotic bodies showed markedly smaller FCS and SSC signals than

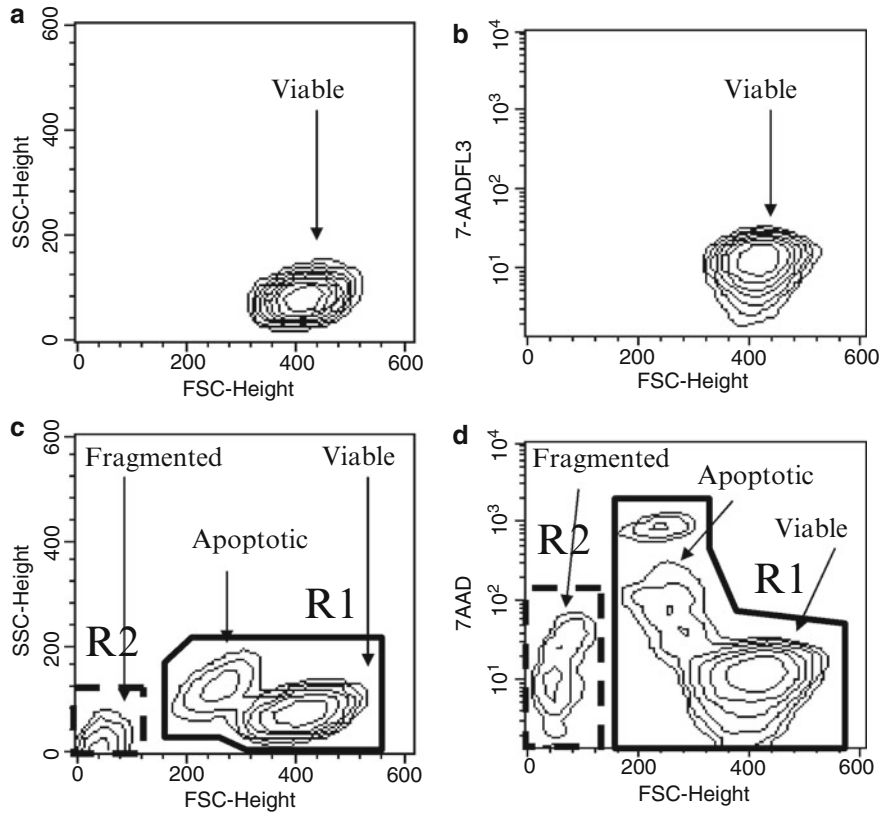

Fig. 1 Flow cytometry approach used to discriminate whole cells from apoptotic bodies by gating in 7AAD/FSC bivariate dot plots. Freshly purified CD19⁺ lymphocytes were labeled with CD19-APC, annexin V-FITC, and 7AAD. Flow cytometry analysis was performed prior to and after 24 h of culture. The experiment was repeated six times. *Panels* (**a**) and (**b**) show SSC/FSC and 7AAD/FSC bivariate contour plots of freshly purified B-cells. *Panels* (**c**) and (**d**) show how whole cells (R1) were differentiated from apoptotic bodies (R2, 7AAD⁻, and lower FSC signal than the lower limit of the 7AAD⁺ apoptotic cells) through combined analysis of the FSC/SSC/7AAD characteristic of the events measured

did live or apoptotic whole cells independently of their occasionally weak 7AAD staining (panels c and d). Thereafter, an event was considered to correspond to a whole cell when it provided an FSC signal greater than the lower limit of 7AAD⁺ apoptotic cells (insert of continuous line boxes in bottom panels). Using these criteria, whole apoptotic cells were clearly distinguishable from apoptotic bodies (inserts of discontinuous line boxes).

1.3 Apoptosis Quantification

Most authors currently quantify the frequency of phenotypically defined apoptotic cells after calculating the apoptotic index (AI), i.e., the percentage of apoptotic cells displaying a specific LAg within a population of cells that remain unfragmented and retain the expression of the LAg [18–20]. However, this approach has two major limitations. Firstly, apoptotic cells fragment into apoptotic bodies that later disintegrate. This leads to an underestimation

of the percentage of apoptotic cells if the debris is excluded from the gates for cell analyses, or, alternatively, to the overestimation of apoptosis if several apoptotic bodies derived from a single cell are misinterpreted as individual apoptotic cells [21]. Secondly, apoptotic cells frequently lose, partially or even completely, the cell surface expression of the LAg used for the identification of specific cell subsets [22–24]; this means that the apoptotic cells from one phenotypically defined cell subset that lose the expression of their characteristic LAg can no longer be identified as targets in the apoptosis quantification, which leads to miscalculations [25].

The limitations of current flow cytometric approaches for evaluating apoptosis warrant the development of a new multiparameter method that (1) identifies and quantifies cells suffering apoptotic lesions in earlier stages of apoptosis; (2) discriminates live, necrotic, and apoptotic cells in a time frame within the death program that is well ahead of LAg loss and the generation of cell debris; and (3) extends AI to provide an estimate of the number of cells that have undergone apoptosis and its relation to the number of seeded cells: the apoptotic rate (AR).

The AR overcomes the limitations of current flow cytometric techniques which do not use internal standards to determine absolute numbers. In previously described methods [6, 7, 9–11], AI has been used to measure the proportion of apoptotic cells in relation to the total number of detectable cells in the test tube at the end point of the cell culture assay. The enumeration of apoptotic cells by the AR reflects the proportion of cells that have undergone apoptosis in relation to the total number of cells seeded at the start point of the cell culture assay. This makes the estimation of the incidence of apoptosis more valid, since current methods ignore late apoptotic cells which have suffered LAg loss or fragmentation into apoptotic bodies. Therefore, the AR is a more sensitive indicator of apoptosis than the widely used AI.

The ability to accurately and sensitively determine the number and population of cells undergoing apoptosis will allow great advances in evaluating new therapies targeted at inducing or inhibiting apoptosis. In addition, it could provide an early marker of therapeutic outcome, enabling clinicians to quickly determine if, for example, a new chemotherapeutic agent is successfully targeting neoplastic cells or if these are resistant to the therapy. The ease of use of flow cytometric techniques allows apoptosis to be used as a clinical parameter. Well-defined interpretations of results such as AR will help develop the use of apoptosis as a marker in making clinical decisions.

A limitation of the proposed method is that AR can only be properly applied in time frames in which the in vitro cell proliferation does not alter significantly the number of cells in the culture. This time frame depends on the rate of proliferation of the studied cells. If the cells do not proliferate (i.e., B-chronic lymphocytic

leukemia cells) then apoptosis can be measured by AR at 24 h or even 48 h of culture. When cells proliferate vigorously (i.e., certain tumor cell lines) it is necessary to perform the apoptosis assays after shorter periods of culture (3–6 h) to avoid interference of proliferative processes on the quantification of cell loss by apoptosis. In any case, even in conditions in which apoptosis and growth simultaneously occur, methods that enumerate apoptotic cells provide more information than those which only provide relative proportions of apoptotic cells.

In summary, apoptosis cannot be accurately quantified by simply taking into account the percentage of cells that show apoptotic lesions. Single-cell approaches must therefore be used with care if occurrence of apoptosis is to be accurately evaluated, and should take into account absolute cell enumeration via the use of an internal microbead standard and the calculation of the AR.

2 Materials

2.1 Equipment

1. Sterile 50 ml conical tubes.
2. 5 ml polystyrene round-bottom tubes 12 × 75 mm.
3. 96-Well flat-bottom culture plates.
4. Neubauer chamber.
5. FACSCalibur flow cytometer (Becton & Dickinson Biosciences).

2.2 Reagents

1. Complete medium: RPMI 1640 supplemented with 10 % heat-inactivated fetal calf serum, 25 mM Hepes, and 1 % penicillin-streptomycin.
2. 7-Aminoactinomycin D (7-AAD)—Highly toxic.
3. Ca^{2+}-binding buffer: Hepes 10 mM, NaCl 150 nM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, and KCl 5 mM; pH adjusted to 7.4.
4. Annexin V-binding buffer containing Ca^{2+}: Hepes 10 mM, NaCl 150 nM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, and KCl 5 mM; pH adjusted to 7.4.
5. Annexin V-FITC.
6. 6 μm CALIBRITE microbeads (Becton & Dickinson Biosciences).
7. Gelatin.
8. Trypan blue.
9. 0.5×10^{-6} M Staurosporine—Highly toxic.
10. 10^{-3} M Cycloheximide—Highly toxic.
11. 2 μg/ml Phytohemagglutinin—Highly toxic.
12. T Cell Expander (Dynal, Oslo, Norway).

3 Methods

The methods described below outline (1) preparation of the microbeads, (2) preparation of the cell suspension, (3) preparation of the culture, (4) preparation of the basal condition, (5) acquisition of cells after culture, and (6) calculation of the apoptotic rate.

3.1 Preparation of the Microbeads

One of the major problems of the use of microbeads in flow cytometric enumeration of cells is the possibility of adherence. Due to this, we need to block the adherence of microbeads to both the tube and the own microbeads by using gelatin in the solution used to dilute the microbeads. Another factor is the sedimentation of microbeads in the tube. Just before adding the microbeads to the cell sample, we need to vortex vigorously the microbead solution.

1. In a 50 ml conical tube, prepare a volume (μl) of Ca^{2+}-binding buffer equal to 100 × number of sample tubes you will use (*see* **Note 1**).
2. Add gelatin 0.05 % (w/v).
3. Heat the tube in a thermal bath to 37 °C for 30 min.
4. Keep at room temperature for 30 min.
5. Add CALIBRITE microbeads to the 50 ml tube to prepare a 1/100 (v/v) dilution.
6. Vortex the 50 ml tube during 1 min.
7. Store at 4 °C in a refrigerator until use.

3.2 Preparation of the Cell Suspension

A cell suspension of tumor cells in complete medium must be obtained. This suspension could be homogeneous (i.e., tumoral cell line) or heterogeneous (i.e., peripheral blood mononuclear cells from a patient suffering from leukemia or tumor cells obtained from a tumor biopsy). All the protocol must be performed using sterile material in a laminar flow chamber.

1. Take 20 μl of the cell suspension and dilute it with 20 μl of trypan blue (*see* **Note 2**).
2. Mix gently and count the viable cells (cells without blue staining) in a Neubauer chamber.
3. Adjust the cells to a cell concentration of 0.5×10^6 viable cells/ml.

3.3 Preparation of the Culture

1. Add 100 μl of complete medium into three wells (triplicate) in 96 flat-bottom culture plates (*see* **Note 3**) and into three 5 ml polystyrene round-bottom tubes.
2. Add 100 μl of the diluted cells to the wells with complete medium (*see* **Note 4**) and into three 5 ml polystyrene round-bottom tubes to make the basal condition.
3. Culture the plate at 37 °C in 5 % CO_2 (*see* **Note 5**).

3.4 Preparation of the Basal Condition

1. Add to the tubes a combination of monoclonal antibodies labeled in FL-2 (i.e., phycoerythrin) and FL-4 (i.e., allophycocyanin) (*see* **Note 6**).
2. Incubate cells at 4 °C in the dark for 20 min.
3. Centrifuge cells at 300 × *g* and 4 °C for 5 min and decant the supernatant (*see* **Note 7**).
4. Resuspend cells and add 100 μl of Ca^{2+}-binding buffer.
5. Add 6 μl of annexin V-FITC diluted 1/5 in Ca^{2+}-binding buffer at 4 °C in the dark for 10 min.
6. Add 100 μl of the prepared microbeads (remember to make a vigorous vortexing of the microbead solution before adding it to the cell suspension).
7. Add 100 ml of 7AAD diluted in Ca^{2+}-binding buffer to a final concentration of 2.5 μg/ml and wait for 3–5 min (*see* **Note 6**).
8. Acquire the cell tubes in a four-color flow cytometer (*see* **Note 8**). You must make a cell gate around microbeads and adjust the number of acquired microbeads (i.e., 2,000 microbeads) to simplify the calculations to obtain the apoptotic rate.

3.5 Acquisition of Cells After Culture

1. Take out the volume of each well with a micropipette (*see* **Note 9**) and add it into 5 ml polystyrene round-bottom tubes.
2. Prepare the 24-h condition like Subheading 3.4 (*see* **Note 10**).

3.6 Calculation of the Apoptotic Rate

The calculation of apoptotic rate (AR) consists of two sequential steps. First, the number of events corresponding to cells which have finished the apoptotic process and have undergone fragmentation into apoptotic bodies or have completely lost the expression of surface markers is calculated from the difference between the number of events corresponding to seeded cells and that of cells which remain in culture and are LAg^+ after challenge. Second, we sum to this number the number of events corresponding to annexin V^+ cells and calculate the apoptosis occurrence with respect to the total number of seed cells:

1. NFC = NSC – NRC
   where NFC = events corresponding to fragmented cells or that completely lost the expression of their LAg; NSC = events corresponding to seeded cells; and NRC = events corresponding to the remaining cells, which include both annexin V^+ and annexin V^- cells.
2. The AR is then calculated by the following equation (*see* **Notes 11** and **12**):

$$AR = \left(NAV^{+}C + NFC\right) / NSC$$

   where NAV^+C = events corresponding to the number of annexin V-positive cells.

4 Notes

1. Prepare an extra 10 % more volume that you will need to be sure that you will have enough volume for all the tests if any problem arises. The minimum volume you must prepare is 10 ml because less volume cannot be shaken properly. Do not use microbeads prepared 10 or more days ago.
2. If you have an excessive number of cells per count chamber field you can dilute the cells in trypan blue until obtaining the proper dilution. A cell count per field between 30 and 120 cells allows accurate counting.
3. This is to measure spontaneous apoptosis. You can induce apoptosis by several kinds of apoptogens like etoposide, staurosporine, or cycloheximide or even study the activation-induced cell death induced by phytohemagglutinin or microbeads coated with anti-CD3 and anti-CD28 antibodies.
4. Critical step: It is very important to seed cells carefully because it affect so much to cell enumeration.
5. The time of culture should be adjusted depending on the apoptosis and cell growth properties of the tumor cells in culture. If the cells grow quickly then the time frame to measure apoptosis should be sorter.
6. If you want to make several different labeling of the cells with different combinations of antibodies you must prepare three culture wells and three 5 ml polystyrene round-bottom tubes for each combination to assess the precision of apoptosis measurement. If you do not want to label with 7AAD then you can add an additional monoclonal antibody labeled in FL-3 channel (i.e., peridinin chlorophyll protein conjugate).
7. Critical step: It is very important to decant cells carefully because it affect so much to cell enumeration due to cell loss.
8. You can label the cells with more or less fluorochrome-labeled antibodies depending on the technical characteristics of your flow cytometer.
9. Critical step: It is very important to take out cells carefully because it affects so much to cell enumeration due to cell loss. You must take out all the volume of the well.
10. It is critical to use for all the tests for a given experiment the same microbead solution for the reference of the ratiometric enumeration.
11. It should be noted that you can calculate the AR of a cell subpopulation defined by the expression of a cell marker and not only the total AR.

12. It is possible to make a more immediate calculation of AR using the next equation:

$$AR = (NSC - NVC) / NSC$$

where
NSC = events corresponding to seeded cells.
NVC = events corresponding to viable cells after culture (number of annexin V-negative cells).

References

1. Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555–556
2. Russell JH, Dobos CB (1980) Mechanisms of immune lysis. II. CTL-induced nuclear disintegration of the target begins within minutes of cell contact. J Immunol 125:1256–1261
3. Cohen JJ, Duke RC (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 132:38–42
4. Khong HT, Restifo NP (2002) Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nat Immunol 3: 999–1005
5. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murakami T, Traganos F (1997) Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 27:1–20
6. Herault O, Colombat P, Domenech J, Degenne M, Bremond JL, Sensebe L, Bernard MC, Binet C (1999) A rapid single-laser flow cytometric method for discrimination of early apoptotic cells in a heterogeneous cell population. Br J Haematol 104:530–537
7. van Engeland M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM (1998) Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31:1–9
8. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger CPM (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184:39–51
9. Gorczyca W, Gong J, Darzynkiewicz Z (1993) Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. Cancer Res 52:1945–1951
10. Gong J, Traganos F, Darzynkiewicz Z (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal Biochem 218:314–319
11. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV (1992) Dead cell discrimination with 7-aminoactinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry 13:204–208
12. Pantaleo G, Graziosi C, Demarest JF, Butini L, Montroni M, Fox CH, Orenstein JM, Kotler DP, Fauci AS (1993) HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. Nature 362: 355–358
13. Ashkenazi A, Dixit VM (1998) Death receptors: signaling and modulation. Science 281:1305–1308
14. Koopman G, Reutelingsperger CPM, Kuijten GA, Keehnen RM, Pals ST, van Oers MH (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84:1415–1420
15. Devaux PF (1991) Static and dynamic lipid asymmetry in cell membranes. Biochemistry 30:1163–1173
16. Zachowski A (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J 294:1–14
17. Fadok VA, Voelker DR, Campbell PA, Bratton DL, Cohen JJ, Noble PW, Riches DW, Henson PM (1993) The ability to recognize phosphatidylserine on apoptotic cells is an inducible function in murine bone marrow-derived macrophages. Chest 103:102
18. Potten CS (1996) What is an apoptotic index measuring? A commentary. Br J Cancer 74: 1743–1748
19. Darzynkiewicz Z, Traganos F (1998) Measurement of apoptosis. Adv Biochem Eng Biotechnol 62:33–73

20. Darzynkiewicz Z, Bedner E, Traganos F, Murakami T (1998) Critical aspects in the analysis of apoptosis and necrosis. Hum Cell 11:3–12
21. Prieto A, Díaz D, Barcenilla H, García Suárez J, Reyes E, Monserrat J, San Antonio E, Melero D, de la Hera A, Orfao A, Álvarez Mon-Soto M (2002) Apoptotic rate: a new indicator for the quantification of the occurrence of apoptosis in cell culture. Cytometry 48:185–193
22. Potter A, Kim C, Golladon KA, Rabinovith PS (1999) Apoptotic human lymphocytes have diminished CD4 and CD8 receptor expression. Cell Immunol 193:36–47
23. Philippé J, Louagie H, Thierens H, Vral A, Cornelissen M, De Ridder L (1997) Quantification of apoptosis in lymphocyte subsets and effect of apoptosis on apparent expression of membrane antigens. Cytometry 29:242–249
24. Diaz D, Prieto A, Barcenilla H, Monserrat J, Prieto P, Sanchez MA, Reyes E, Hernandez-Fuentes MP, de la Hera A, Orfao A, Alvarez-Mon M (2004) Loss of lineage antigens is a common feature of apoptotic lymphocytes. J Leukoc Biol 76:609–615
25. Prieto A, Reyes E, Diaz D, Hernandez-Fuentes MP, Monserrat J, Perucha E, Munoz L, Vangioni R, de la Hera A, Orfao A, Alvarez-Mon M (2000) A new method for the simultaneous analysis of growth and death of immunophenotypically defined cells in culture. Cytometry 39:56–66

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