A Rapid and Simple flow Cytometric Method for Measuring Cell Viability Using Propidium Iodide Staining and Forward Scatter Measurement

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***Background*: The importance of the determination of cell viability has prompted the development of several assays of viability that utilize the exclusion of certain dyes by viable cell membranes. Recently, flow cytometry has been adapted to estimate cell viability by using fluorescent dye which is excluded by living cells on the basis of altered dead cell properties.**

***Objective*: We have developed a flow cytometric method for measuring cell viability after staining with propidium iodide (PI) and have compared it with the classical colorimetric method, MTT assay, which is currently widely used in cytotoxicity assays in the research field.**

***Methods*: We performed flow cytometry and MTT assay for the comparison of the sensitivity of the assessment of cell viability.**

***Results*: Decrease of cell viability was measured by flow cytometry with the addition of as little as 0.002% Triton-X 100 in comparison to MTT assay which could only reveal a similar decrease of cell viability with the new method to 0.008% Triton-X 100.**

***Conclusion*: Our results demonstrate this new method to be more sensitive and simple for the assessment of cell viability. (Ann Dermatol 8:(3)195~200, 1996)**

Key Words : Cell viability, Flow cytometry, MTT, Propidium iodide

The determination of cell viability is important in cell culture studies, and there are several assays of viability that utilize the exclusion of certain dyes by viable cell membrane [1]. Although trypan blue exclusion is the common test for cell viability, it is inconvenient to count large number of samples at the same time. MTT assay is one method of assessing cell viability and is commonly used in cell viability tests with a large number of samples [2]. This rapid and simple assay method has been widely used in medical and biological fields, especially in immuno-cytotoxic activity studies or cytotoxicity testing. Since the publication of the original method, there have been numerous modifications, none of which have been used without limitations [3]. Flow cytometry is a technique utilized for the rapid measurement of particles or cells as they flow, one by one, in a fluid stream through a counter. A wide range of measurable cellular parameters such as nucleic acid content, enzyme activity, calcium flux, membrane potential and pH can be measured by flow cytometry [4]. Flow cytometry has been used to identify dead cells simply by using fluorescent dyes which are excluded by living cells [5].

We have developed a sensitive flow cytometric

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method for measuring cell viability after propidium iodide (PI) staining, analyzing the parameters of red fluorescence of propidium iodide (PI) versus forward light scatter (FSC). Sensitivity of this new flow cytometric method was compared to that of MTT assay, which is a frequently used colorimetric method.

MATERIALS AND METHOD

Cell lines

A human melanoma cell line, G361, was obtained from American Type Tissue Collection (ATCC) (Rockville, MD) through the Korean Cell Line Bank (Seoul, Korea). Cells were maintained in RPMI 1640 (Gibco, Grand Island, NY) and supplemented with 10% fetal calf serum.

Cell suspensions

Cultured cells were exposed to Triton X-100 for 20 hr. The final concentration of Triton-X 100 ranged from 0.001% to 0.016%. The cells were washed and dislodged by treatment with 0.1% trypsin (Gibco, Grand island, NY) and neutralized by trypsin inhibitor (Gibco, Grand island, NY). Cells were then collected and processed.

Flow cytometry

After the cells had been serially exposed to Triton-X 100 for 20 hr, 50 μl of PI (50 mg/ml) (Sigma, St.Louis, MO) was added to the cell suspensions for the purpose of staining cells to be analyzed by flow cytometry which was performed with a Facstar plus (Becton-Dickinson, San Jose, CA). PI fluorescence was measured together with forward light scatter (FSC). All data was recorded and analyzed using LYSIS II software (Hewlett-Packard, CA). At least $5X10^3$ cells were analyzed and all measurements were done under identical instrument settings.

MTT assay

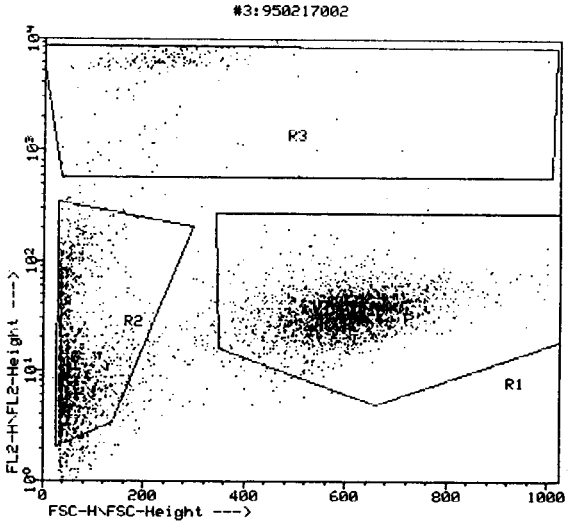
Before exposure to Triton-X 100, $1X10^4$ cells were inoculated onto a flat-bottomed 96-well plate and cultured for 24 hr at 37℃ in 5% CO_2. After the cells were exposed to Triton X-100 for 16 hr, 20 μl of MTT (5 mg/ml) (Sigma, Ts.Louis, MO) was added to each well. The cells were incubated for a further 4 hr at 37℃. The supernatant was removed, and 200 μl of dimethylsulphoxide (DMSO) was added to each well to dissolve the formazan products. Absorbance was determined spectrophotometrically at 570 nm using an ELISA reader (Titertek Multiskan MCC/340, Labsystem, Finland). The results were expressed as a percentage of the control.

RESULT

Flow cytometric analysis of cell viability

Figure 1 shows a typical 2-parameter frequency contour plot of forward scatter (FSC) versus red fluorescence of propidium iodide (PI) for G361 human melanoma cell lines. Three cell subpopulations were identified and designated as regions 1 (R1), 2 (R2) and 3 (R3). Most undamaged cells appeared in R1, exhibiting high FSC and low PI red fluorescence (FL) (Fig. 1). With an increased concentration of Triton-X 100, cells were found to migrate from R1 to R3, exhibiting low FSC and high FL (Fig. 2).

Also identified was an additional subpopulation,



#3:950217002

----- Region Stats -----

File: #3:950217002 Sample: 066

Date: 2/16/95 Ungated Sample

Parameters: FSC-H(LIN),FL2-H(LOG)

Total= 5000 Gated= 5000

Rgn	Events	% Gated	% Total
1 R1	2672	53.44	53.44
2 R2	1831	36.62	36.62
3 R3	227	4.54	4.54

Fig. 1. Flow cytometric analysis of G361 melanoma cells. Two dimensional frequency contour plots of forward scatter (FSC) versus fluorescence of propidium iodide (FL) of untreated cells. R1, R2 and R3 represent each cell subpopulation on cell scatter.

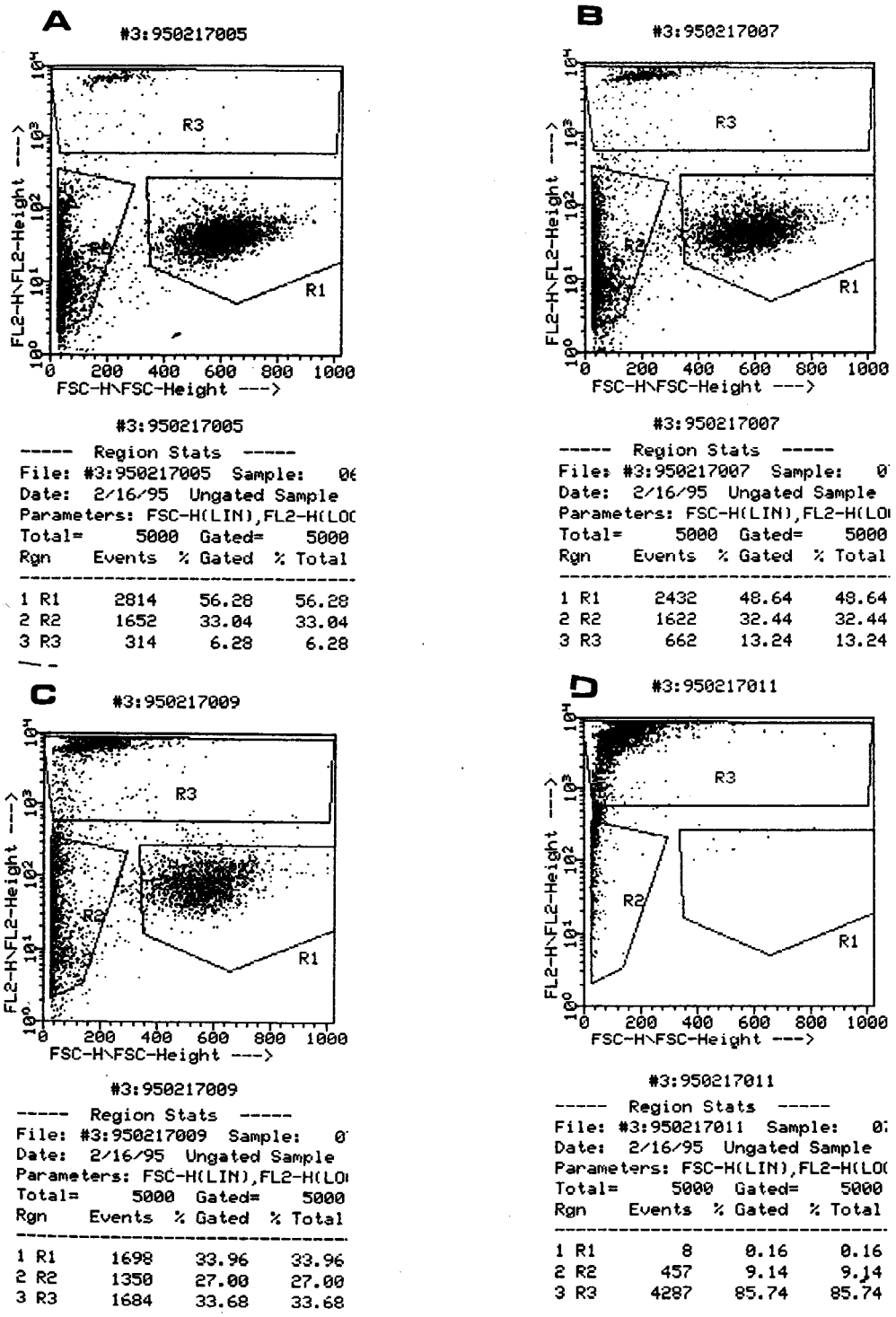


Fig. 2. Dose dependence uptake of PI by G361 melanoma cells. G361 melanoma cells incubated with Triton-X 100 (0.002 - 0.016%) for 20 hr. Dot-plot on flow cytometry of FL (fluorescence of propidium iodide) vs. FSC (forward scatter) are shown after incubation with Triton-X 100 with the following concentration: A) 0.002% B) 0.004% C) 0.008% D) 0.016%. At low concentration of Triton-X 100, R3 cells occupy only about 5% in total cells but extend to 75% at high concentration. However R2 cells decrease slowly from about 40% to 20% of total cells in contrast to increasing of Triton-X 100.

Table 1. Comparison of cell viability tested by flow cytometry and MTT assay

Method	Exp.NO.	Triton-X 100 concentration(%) 0.0	0.001	0.002	0.004	0.008	0.016
FCM	1(N=3)	*100.0±11.7	91.0±29.2	70.1±11.5	38.4±7.4	8.9±0.2	0.02±0.001
	2(N=3)	100.0±18.4	98.2±13.9	86.7±7.9	63.4±0.5	14.9±3.8	0.01±0.002
	total	100.0±12.6	95.4±26.1	78.1±12.7	50.6±15.1	11.9±4.4	0.01±0.001
MTT	1(N=3)	100.0±10.2	107.2±12.6	107.4±9.8	95.5±5.4	74.4±17.2	21.9±7.5
	2(N=3)	100.0±3.5	98.1±4.8	98.8±3.9	96.5±6.2	56.4±3.1	4.7±0.4
	3(N=3)	100.0±3.6	101.6±5.6	103.6±5.6	103.6±4.4	64.5±5.6	4.4±0.8
	total	100.0±5.7	102.4±4.6	103.4±4.3	97.4±2.4	65.2±9.1	10.5±99

FCM=flow cytometry, MTT=3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide
*negative control data regarded as 100 percent(%±S.D.)

designated R2, which accounted for approximately 40% of total cells (Fig. 1) and fluctuated according to the concentration of Triton-X 100 (Fig. 2). This region exhibited low FSC and low FL, suggesting a maintained ability to exclude propidium, but decreased size of cells compared to R1. Based on these observations, we concluded R2 to represent a group of cells which, though damaged, still retain the ability to exclude propidium. These R2 group cells could be excluded from debris or floating materials according to a decreasing proportion of R2 at highly damaged state on flow cytometry (Fig. 2).

To compare these results with a MTT assay, we tentatively defined the ratio of R1/R3 as an index of cell viability. We also analyzed by our results adopting the ratio of (R1+R2)/R3 (data not shown), but could not find any significant difference from R1/R3 which revealed decreased viability of G361 cells from 0.002% of Triton-X 100 (Table 1) (Fig. 3A).

MTT assay of cell viability

MTT assay was unable to detect any significant change until the addition of 0.004% of Triton-X 100 to the culture medium. With the addition of 0.008% Triton-X 100 to the culture medium, cell viability of G361 cells decreased to 65% of control level (Table 1) (Fig. 3B).

DISCUSSION

The determination of cell viability is essential to tissue culture and cytotoxicity study. There are several accepted assays of viability that utilize the exclusion of certain dyes by live cell membranes [1], trypan blue exclusion being the most common test for cell viability. However, it may be inaccurate in the identification of dead cells as cells must be counted within 3-5 min because the number of blue staining cells increases with time, as shown by Hudson et al [6]. Time is less critical when eosin Y is used, but the identification of dead cells is more subjective [7]. When large numbers of samples have to be counted, it may be inconvenient to perform all the tests on the same day and a rapid and more reliable method is needed.

Certain fluorescent dyes have been found to be more reliable indicators of cell viability than the more traditional colored dyes [8]. Fluorescent dyes such as ethidium bromide and propidium iodide (PI) are known to pass through the membranes of dead or dying cells [9]. Double staining with acridine orange and ethidium bromide is commonly performed on cell suspensions and cells can be evaluated by flow cytometry or fluorescent microscope [1,5]. The use of flow cytometry has the advantage that large numbers of cells can be counted quickly and that the determination of negative/positive is objective.

Recently, an improved method to determine cell viability by simultaneous staining with fluoresein diacetate-propidium iodide has been reported to be a rapid, convenient, and reliable method of determination of cell viability. Because it is known that dead cells usually show decreased for-

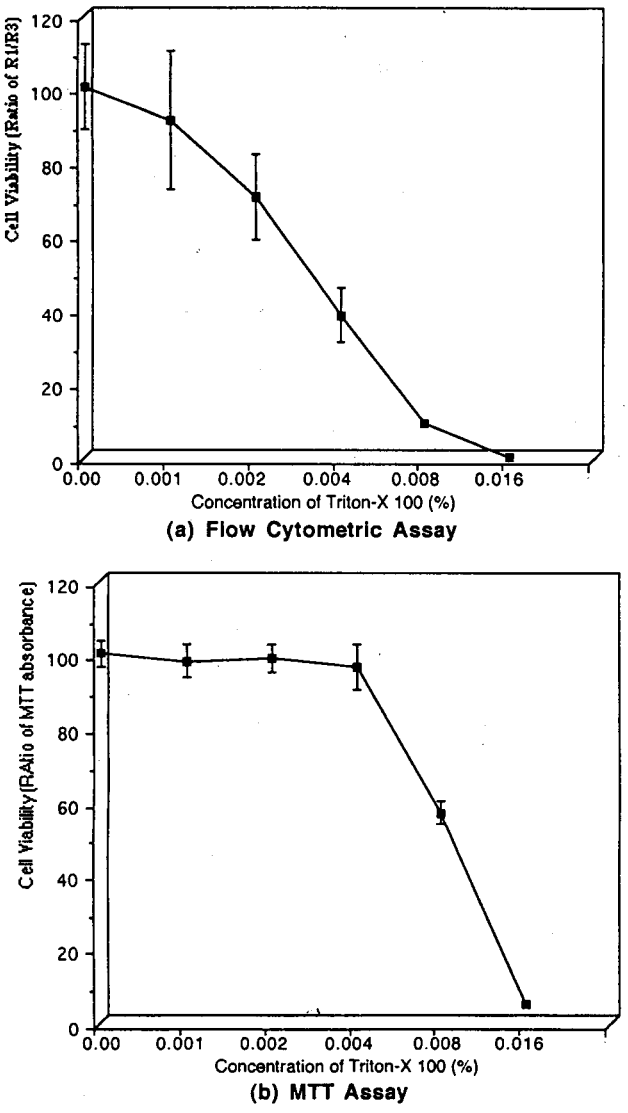


Fig. 3. A) Flow cytometric assay for cell viability. The change of cell viability begins from 0.002% of Triton-X 100 treated state. B) MTT assay of cell viability. The change of cell viability is minimal until the concentration of Triton-X 100 reaches 0.008%.

ward light scatter (FSC) [5], we have attempted to develop a new flow cytometric method for measuring cell viability after PI staining, analyzing two parameters, red fluorescence of PI versus forward light scatter. Dive et al [10] described the presence of a transitional state of cell damage by multiparametric analysis of cell membrane permeability by two color flow cytometry with complementary fluorescent probes. A third population whose fluorescence characteristics matched neither those of living or dead cells was observed which, displayed decreased FSC characteristics. So the adaptation of adapting forward light scatter, may prove useful to analyze cell viability even without other fluorescent dyes [11].

In our experiments, three cell subpopulations were identified. Most undamaged cells appeared in R1, exhibiting high FSC and low red fluorescence of PI (FL). By increasing Triton-X 100 concentration, most cells moved to R3, exhibiting low FSC and high FL (Fig. 1). Also identified was an additional population, R2, exhibiting low FSC and low FL. These characteristics suggest a continued facility to exclude propidium but decreased size of cells compared to R1. In fact flow cytometry showed that R2 decreased approximately from 40% to 20% in the total cell population, related to the concentration of Triton-X 100 added, when concentration of Triton-X 100 became higher than 0.016% (Fig. 2). One possible understanding of these results needs the making of a definition of the composition of R2 cells. We propose that there may be not only cell-debris or floating particles in R2, but also damaged cells as Dive et al [10] argued in their article. Another understanding is the hypothesis that cell death requires a transitional state of damaged cell from the healthy cell state. So we propose that there might be a pathway from R1 to R3 through R2 in going to cytotoxic mechanisms. However in this experiment, for the purpose of a definite cell viability, we tentatively defined the ratio of R1/R3 as an index of cell viability and cell viability analyzed by flow cytometry decreased from 0.002% of Triton-X 100 (Fig. 3A).

We compared our results with a MTT assay, which was first described by Mosmann 2 in 1983. MTT assay is based on the conversion of yellow tetrazolium salt, 3-(4,5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), to the colored formazan product by mitochondrial enzymes in viable cells [3]. The concentration of the colored product can be measured spectrophotometrically and is proportional to the number of viable cells. There have been numerous modifications to improve the sensitivity of this assay, none of which have proved completely satisfactory [3], but some reporters mentioned the disadvantage of MTT assay in cell viability analysis [12].

Our results by the new flow cytometric method

were compared with those by MTT assay, which is a frequently used colorimetric method. MTT assay revealed no significant difference until the addition of 0.004% of Triton-X 100 to culture medium. After addition of 0.008% Triton-X 100 to culture medium, cell viability of G361 cells decreased to 65% of control level (Fig. 3). These results show that cell viability analysis by flow cytometry is much more sensitive than MTT assay .

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