Comparison of the Automated Fluorescence Microscopic Viability Test With the Conventional and Flow Cytometry Methods

Jang Su Kim,¹ Myung Hyun Nam,¹ Seong Soo A. An,^{2,3} Chae Seung Lim,^{1,2*}

Dae Sung Hur,⁴ Chanil Chung,⁴ and Jun Koon Chang⁴

¹Department of Laboratory Medicine, Seoul, Republic of Korea

²Brain Korea 21 Program for Biomedical Science, College of Medicine, Korea University, Seoul, Republic of Korea

³Gachon Bionano Research Institute, College of Bionano Technology, Kyungwon University, Seoul, Republic of Korea

⁴Nanoentek Corporation, Seoul, Republic of Korea

The cell viability test is an essential tool in any laboratory, performing cell-based studies and clinical laboratory tests. The trypan blue exclusion method is the most popular assay for its simple concept among various diagnostic tools. However, several disadvantages include time-consuming and labor-intensive steps with low precision. In this study, we evaluated a new technique for the automatic cell viability measurement with microscopic cell counter and microchip. Upon blood draw from 11 healthy volunteers, Mononuclear cells were separated immediately from the heparinized whole blood, and the viable cells were diluted from 100 to 1%. The cell viability tests were performed simultaneously with following three methods: the conventional manual trypan blue exclusion method; the

flow cytometry measurement with propidium iodide stain; and the newly developed microscopic cell counter with microchip. Linearities, precisions, and correlations from three methods were analyzed and compared. The correlations data from the microscopic cell counter were in good agreement with both the conventional trypan blue method ($r = 0.99$, $P < 0.05$) and the flow cytometry ($r = 0.99$, $P < 0.05$), respectively. The precision (2.0–6.2%) and linearity from the microscopic cell counter method with microchip were superior in comparison with the conventional method. The microscopic cell counter with microchip performed well with high precision, linearity, and efficient running time than both the manual trypan blue and the flow cytometry methods. *J. Clin. Lab. Anal.* 25:90–94, 2011. © 2011 Wiley-Liss, Inc.

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INTRODUCTION

The cell viability test determines the live cells from the dead ones. Providing overall information of cells' condition, the cell viability test became an essential tool in any laboratory, conducting cell-based studies and performing clinical laboratory tests. The principles of the cell viability test were based on the presence of damaged or unimpaired membranes, where living cells with intact cell membranes could exclude certain dyes, but not by dead cells. Among numerous cell viability tests and methods, the trypan blue exclusion test with hemocytometer is widely used due to its simple procedure. However, this conventional assay revealed several limitations such as lengthy running time and labor-intensive

microscopic examination steps with low precision in handling large numbers of samples (1). Alternatively, when the cell viability test for the flow cytometry was introduced, it performed well with improved reproducibility. But the flow cytometry required a complex and

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Jang Su Kim and Myung Hyun Nam contributed equally to this work.

*Correspondence to: Chae Seung Lim, Department of Laboratory Medicine, College of Medicine, Korea University Guro Hospital, 80, Guro Dong, Guro Gu, Seoul, Republic of Korea.

E-mail: malarim@korea.ac.kr

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expensive instrument with higher maintenance cost per test and lengthy testing time (2). Therefore, development of a simple, rapid, user-friendly, and reliable test for measuring cell viability was sought by many clinical laboratories.

Here, the new cell viability test was presented, which could be performed with the newly developed microscopic cell counter and microchip (Adam, Nanoentek, Seoul, Republic of Korea). This device adopted a direct cell counting method for differentiating viable and nonviable cells on a designed microchip with propidium iodide (PI) stain. After optimizing the newly developed cell viability test, it was compared with the manual trypan blue exclusion and the flow cytometric methods with PI stain.

MATERIALS AND METHOD

Mononuclear Cell Preparation for Viability Test

Five milliliters of heparinized whole blood were collected from 11 healthy volunteers at Korea University Ansan Hospital in Ansan City with informed consent. This study was approved by the institutional review boards of the Korea University Ansan Hospital. Mononuclear cells (MNC) were isolated by centrifugation on Ficoll-Paque centrifuge (Pharmacia Biotech, Uppsala, Sweden). Ten samples were frozen and thawed for the three cell viability tests: the manual trypan blue exclusion method; the flow cytometry test; and the microscopic cell counter with microchip (Adam, Nanoentek). For the preparation of the dead cells as a control parameter for comparison, MNC suspensions were stored at -80°C for 1 day in the absence of cryoprotectant.

Viable and Nonviable Cell Dilution

Freshly isolated MNC and dead cells from above procedures were used to measure the frequency of nonviable cells. After the viability test with the viable and nonviable cell suspension controls, cell suspensions were mixed and adjusted with various viable cell concentrations (1, 25, 50, 75, and 100%). During the time course experiments (from 0 day to 10 day), cell viability of each suspension at given time was also measured. For the determination of reproducible cell concentrations at each point, all three cell viability tests were performed simultaneously within 30 min for a total of 11 time points, as mentioned below.

Trypan Blue Dye Exclusion Test With Hemocytometer

MNCs from the different viability groups were treated with trypan blue stain (100 $\mu\text{l}/\text{ml}$) for 5 min for counting the percentage of dead cells by a light microscope

(Nikon, Tokyo, Japan) and the Neubauer counting chamber. The percentages of dead cells were calculated blindly from a total of 200 cells per sample by the same investigator.

Flow Cytometric Measurement of Cell Viability

Cells were washed once with PBS, and resuspended in fresh growth medium to achieve an initial cell density of $0.5\sim 1 \times 10^5$ cells/ml. For the appropriate time of viability assessment, an aliquot (usually 2 ml) of each MNC suspension was placed in a test tube. In a separate experiment, the viability dye, PI, was added to reach a final concentration of 1 $\mu\text{g}/\text{ml}$. The cells were stained for 3 min at room temperature, then was placed on ice in the dark, and analyzed by Coulter Epics (Becton Dickinson, San Jose, CA). Total of 10,000 events were counted for each sample by flow cytometry.

Portable Microscopic Cell Counter With Microchip for Cell Viability Test

The new cell viability test was developed by modifying (or improving) the previously reported microscopic technique for counting residual WBC with microchannel chip (3,4). The microchannel chip was made with polymethylmetacrylate (PMMA). The two microfluidic channels were located in the center of the microfluidic chips with a width of 75×25 mm in size and a height of 0.1 mm, and the actual channel dimension for sample load was $48 \times 4 \times 0.1$ mm³. Each channel was designed for counting total and dead leukocytes, after the PI staining with and without formalin, respectively. Total sample staining procedure took only 3 min, where the capillary force spread cells automatically throughout the channel cavity. The disposable plastic chip was mounted and analyzed on a tabletop size inverted microscope-type optical system (Adam, Nanoentek) with dimension of $220 \times 375 \times 250$ mm³ and weight of 9 kg. A green LED (4W, Seoul Semiconductor, Seoul, Republic of Korea) with a wavelength of 520~535 nm was used as an excitation fluorescent light source. Emitting fluorescence signals were collected with a $4 \times$ objective lens and passed through a filter block (585~625 nm) to a charge-coupled device camera (1/3 inch sensor, B/W camera, Sunkwang Electronics, Republic of Korea). To ensure the accuracy of cell counting, 60 frames of fluorescence images from each channel, corresponding to 8.5 μl of a neat sample, were processed for counting approximately 1,000–3,000 cell events. The final concentration of viable leukocytes in a sample was determined directly by counting the signals from the microscope-captured images, and the percent of viable cells were calculated from the ratio of viable and dead cells without and with significant intensities from PI staining, respectively.

Statistical Analysis

The data were expressed with mean $\pm$ standard deviation (SD). ANOVA test was performed to compare the results, and a *P*-value of <0.05 was considered to be significant. Linearity was assessed by calculating Pearson's regression coefficient between the observed and the expected values; for the observed values, a mean of 11 observations was used. Precision of the cell viability was defined with the coefficient of variation (CV) at each viability level (4).

RESULTS

Linearity

The linearities of the cell viability tests from three methods were determined over a range of diluted concentrations of viable cells (1, 25, 50, 75, and 100%), as shown in Table 1 and Figure 1. In comparison with the expected values, all three methods showed good linearity with the Pearson's values, higher than 0.99, uniformly.

Correlation of Viability Tests

Significant correlations (in percentage) were observed between all three methods, from the cell viability data with the microscopic cell counter method to the flow cytometry and to the manual trypan blue exclusion methods, respectively (Fig. 1).

Precision

Manual trypan blue cell viability test performed less precise than other measurements from both the flow cytometry and the microscopic cell counter methods. At day 0, the manual trypan blue exclusion method showed a higher CV of 4.3% than both the flow cytometry and microscopic cell counter methods, as shown in Table 2. The flow cytometry and new microscopic cell counter methods revealed sufficient precisions in all expected ranges (intra-assay CV $\leq 15\%$),

except at low concentrations (or cell viability) on the 10th day. High CV ($>20\%$) were observed in all three methods from concentrations below 1.4% viability at day 10 (value determined by the manual trypan blue test as the standard).

Speed of Analysis

Manual trypan blue cell viability test per sample took approximately 15 min, including sample preparation and cell counting. In comparison, the flow cytometric measurement took approximately 15–20 min, causing delays in the analysis. On the other hand, the microscopic cell counter with microchip took just 5 min including all processes (steps).

DISCUSSION

The cell viability test could be used in tremendous number of applications. It could be used in the drug resistance tests for cancer (5) and antibiotics susceptibility test for bacteria and fungi (6). In organ transplantation, it could be applied to monitor the possible complications and rejection of the implanted organ.

Fig. 1. Comparisons of cell viabilities from three methods. High correlations were observed from the trypan blue method with the microscopic cell counter ($r = 0.99$), and with the flow cytometry ($r = 0.99$), respectively (♦ viability from the flow cytometry, □ viability from the microscopic cell counter).

TABLE 1. Comparison of Three Cell Viability Tests Using Artificially Diluted Samples ($n = 11$)

Expected level of cell viability (%)	Observed values						<i>P</i> -value
	Trypan blue		Microscopic cell counter		Flow cytometry		
	Mean±SD	CV (%)	Mean±SD	CV (%)	Mean±SD	CV (%)	
100	96.7±4.1	4.3	98.2±2.0	2.0	97.6±2.5	2.5	<i>P</i> <0.05
75	74.1±7.7	10.3	72.2±2.3	3.2	71.5±19	2.6	<i>P</i> <0.05
50	44.1±2.5	5.6	42.7±1.7	4.0	43.3±2.5	5.7	<i>P</i> <0.05
25	27.1±4.5	16.6	23.1±1.4	5.9	21.8±0.8	3.8	<i>P</i> <0.05
1	2.2±0.8	37.2	1.6±0.4	22.6	1.3±0.3	26.6	<i>P</i> <0.05

TABLE 2. Comparison of Viable-Cell Percentages From the Time Course Study, Determined With the Trypan Blue Exclusion Test, the Microscopic Cell Counter, and the Flow Cytometer

Storage time	Observed values					
	Trypan blue		Microscopic cell counter		Flow cytometry	
	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)	Mean $\pm$ SD	CV (%)
0 day	96.7 $\pm$ 4.1	4.3	98.2 $\pm$ 2.0	2.0	97.6 $\pm$ 2.5	2.5
1 day	91.3 $\pm$ 3	3.3	92.7 $\pm$ 1.8	1.9	91.9 $\pm$ 2.4	2.6
2 day	81.5 $\pm$ 3.3	4.1	79.0 $\pm$ 1.4	1.7	83.4 $\pm$ 4.2	5.1
3 day	74.2 $\pm$ 5.9	7.9	77.0 $\pm$ 3.0	3.9	71.0 $\pm$ 2.9	4.1
5 day	69.2 $\pm$ 4.1	5.9	65.3 $\pm$ 2.5	3.8	67.3 $\pm$ 2.3	3.4
6 day	32.3 $\pm$ 2.7	8.3	33.2 $\pm$ 3.9	11.7	29.7 $\pm$ 2.6	8.8
10 day	1.4 $\pm$ 0.9	65.7	1.2 $\pm$ 0.4	33.3	1.2 $\pm$ 0.2	33.3

It also could measure the possible deleterious effects of the cryoprotectant on hematopoietic cells after thawing from the liquid nitrogen storage (7,8).

Assessment of the cell viability would depend on the cellular properties. The dyes used in correlation experiments could enter the live cells through intact membrane. The trypan blue exclusion test with the hemocytometer was widely used, but carried certain limitations such as low precision and operator dependency. In addition, errors from the pipetting and transferring processes, and cell counts with Neubauer chamber by different operators could increase CV values. Such errors would also be found in the flow cytometric measurements.

Nonviable cells could be stained and evaluated with various kinds of fluorescent dyes such as PI, SYBR-14, YOYO-1, TOTO-1, or acridien orange (9–13). Among them, PI staining was the most popular method for staining mammalian and bacteria cells (10,12,14). Many applications with PI were developed due to its simplicity: the procedure was very easy to learn and to perform, where the stained cells would reveal very bright red colors for the clear identification. However, the cell viability test with PI would require special and expensive instruments such as the fluorescent microscope or the flow cytometry analyzer. Even though PI revealed its superior ability with the flow cytometry for discriminating the dead cells from live ones, whole procedure took closer to 20 min, including time for setting and priming the instrument, and analysis. Their shear size of the instrument could also be a problem for any small laboratories and tables. To solve these problems, an automatic instrument for performing the cell viability test was introduced. For example, Beckman Coulter Vi-Cell (7) was developed from the modified trypan blue method, and produced results with similar precisions in comparison to the manual method.

The new microscopic cell counter with microchip (Adam, Nanoentek, Seoul, Republic of Korea) could

provide a solution to solve above limitations. PI staining method was further developed and improved to produce the rapid results within 5 min, including all procedures (or steps). In addition, the device revealed greater precision and accuracy with the statistical confidences than the manual trypan blue method. In the manual trypan blue stain, only 200 cells were counted to measure their viability, but in Adam, larger number of cells, approximately between 1,000 and 3,000 cells, were counted. Greater number of counted cells and the repetitions of the experiments by the microscopic cell counter and microchip for the analyses would have provided the better statistical significance. The real-time cellular images and archived data could also be used for analyzing other parameters. The microscopic cell counter with microchip demonstrated that the systematic application with the multiple fluorochromes could be engineered in future to display one or more selected classes of cells, macromolecules, or specific organelles in real-time. The cost for viability test would be about \$2 for the disposable microchip, relatively higher than the manual trypan blue test. As the microchip could be easily isolated and disposed, one of the main advantages of the microchip would be in handling hazardous samples, minimizing the contact by the researchers.

In general, the greater need for measuring cell viability in clinical applications would be the characterization of the viable cells for the hematopoietic cell therapy (HCT). Various sources of the Hematopoietic stem cell included bone marrow and cord blood. In this study, normal healthy peripheral MNCs were used to determine their viability. For the detailed applicability of the automatic cell viability method in the HCT, the current method with microscopic cell counter and microchip should be compared with a simple microscopic cell counter method with various conditions, including different cell populations, time, or an effect of other factors.

In conclusion, the new automatic cell viability method with the microscopic cell counter and microchip would

offer many significant advantages for cell-based assays and analyses. This method could also perform rapid cell viability assessment for processing large number of samples.

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