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RAPID DETERMINATION OF THE NUMBER OF VIABLE YEAST CELLS DURING FERMENTATION BY FLOW CYTOMETRY*

Hideaki Endo*[1], Tetsuhito Hayashi*[1], Junko Nakayama*[1], Yoshiyuki Mukada*[1] and Etsuo Watanabe*[1]

Rapid determination of the number of yeast cells by Flow cytometry (FCM) technique was described. A fluorescein diacetate (FDA) was used to distinguish between viable cells and dead cells. The difference in fluorescence scattergram between the both yeast cells was observed. The FCM method provided the determination of cell number during fermentation. The event number of FCM increased corresponding to the cell growth. A good correlation was observed between the values determined by the FCM method and the colony counting method in the range of 10^5–10^8 cells/m*l*. One FCM assay could be completed within 60 s and the total assay time including the preparation of microorganisms was within 30 min.

Key words: Flow cytometry, Yeast, Cell number, Fluorescein diacetate

Introduction

In the yeast fermentation process, the determination of viable cell number is very important to increase the productivity. Direct microscopic counts and colony counts (Postgate, 1969) have been employed to determine the viable cell number as traditional method. Direct microscopic counts is simple. However, it cannot be applied to cell number less than 10^6 cells/m*l* Although colony counts are reliable, the method is time-consuming.

Several new methods have been developed for the rapid determination, such as electrochemical (Matsunaga, *et al.*, 1984; Ramsay *et al.*, 1988), ultrasonic (Zips *et al.*, 1989; Endo *et al.*, 1989), and biosensor (Hoshi *et al.*, 1991). Electrochemical and biosensor methods enable us to determine the number of viable cells in less than 10 min. However, in most cases, these methods cannot be applied to bacterial number less than 10^7 cells/m*l*. The ultrasonic method can be applied to rapid determination but it measures cell density regardless of their vitality.

In recent years, the technique of flow cytometry (FCM) has made significant contributions to studies in several areas of biology, including medicine, cytology, immunology and biotechnology. FCM combines the advantages of microscopy and biochemical analyses for the measurement of physical and biochemical characteristics of individual cells. In this method, cell particles in suspension flow in single file at a uniform speed through a laser light beam with which they interact individually. This yields, for each cell, a light scattering pattern which provides information about cell size, shape, density, and surface morphology. Furthermore, fluorophore labeling of cells and subsequent measurement can give quantitative data on specific target molecules or subcellular constituents and their distribution in the population (Al-Rubeai and Emery, 1993). Although FCM has been used primarily for studying eukaryotic cells, it was recently applied to microorganisms (Miller and Quarles, 1990; Diaper *et al.*, 1992; Urano *et al.*, 1993; Magarinos *et al.*, 1997). We have also applied FCM technique to several species of bacteria (Endo *et al.*, 1997; Endo *et al.*, in press).

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*[1] Department of Food Science and Technology, Tokyo University of Fisheries, 5–7, Konan 4-chome, Minato-ku, Tokyo 108–8477, Japan (東京水産大学食品生産学科).

On the other hand, fluorescein diacetate (FDA) is often used to discriminate viable cells from their dead. (Chzanowaki *et al.*, 1984; Lang *et al.*, 1993). This dye is lipophilic and can pass into the cell through the membrane. Once FDA enters the cells, it is hydrolyzed by the cytoplasmic esterases of living cells and the resulting compound fluoresces green.

In this paper, we describe the following applications of FCM technique; 1) Use of FDA for the distinction between viable and dead yeast cells, 2) Application of FCM for the determination of viable cell number during yeast fermentation, 3) Investigation of the correlation between the proposed FCM method and the colony counting method.

Materials and Methods

Reagents

FDA ($C_{24}H_{16}O_7$) was purchased from Sigma (Mo, U.S.A.). Peptone and yeast extract were obtained from Difco laboratories (Michigan, U.S.A.). Other chemicals were purchased commercially and were of analytical reagent grade.

Microorganism and cultivation

Sake yeast No. 7 (*Saccharomyces cerevisiae*) was chosen as a model microorganism for fermentation. This strain was cultivated in YEPD medium containing (g/*l*); yeast extract (10), peptone (20), glucose (20) using a shaking flask at 30℃ for 20 h.

Fermentation system

Biopak (Yamatake & Co., Ltd., Tokyo, Japan) was used for yeast fermentation process. This system consisted of a jar fermentor, an oxygen electrode, a pH electrode, and a personal computer. The cells were grown at 30℃ in 2 *l* of broth containing the above ingredients in the fermentor (400 rpm agitation, 2 vvm aeration). Several parameters such as dissolved oxygen concentration (DO), pH, temperature, and agitation were monitored by the system.

Sample Preparation

1) Sample of yeast cells

Yeast cells were grown to the exponential phase of growth. The cells were harvested by centrifugation (8,500×*g*, 2 min), washed (8,500×*g*, 2 min) with 0.9% NaCl solution, and pelleted. The washed cells were suspended in 0.9% NaCl solution and adjusted to appropriate concentration (10^3–10^6 cells/m*l*) for the preparation of calibration curve. As a reference sample, the dead cells of yeast prepared by heating (90℃ for 20 min) were suspended in 0.9% NaCl solution as described above procedure.

In the fermentation process, a portion of a broth was derived from the jar fermentor. The cells were prepared with same procedure described above.

2) Staining procedure

A 0.5 m*l* of the FDA suspended in 0.1 M Tris–HCl buffer (pH 7.4) was added to 0.5 m*l* of cell suspensions (final concentration of FDA: 1, 10, 20, or 50 μg/m*l*). The samples were incubated at 25℃ for 30 min.

FCM Measurement

Flow cytometry was performed using EPICS XL (Coulter Co., Florida, USA) equipped with an argon laser. The power output was 15 mW and the 488 nm wavelength was used excitation. Light scattered in the forward light scatter was filtered by a 488 nm narrow band-pass filter and collected by photomultiplier. Fluorescent light was collected in the orthogonal direction and detected using four

kinds of photomultipliers: FL1 (505–545 nm); FL2 (560–590 nm); FL3 (605–635 nm); and FL4 (660–700 nm).

After excitation at 488 nm, FDA was detected at 505–545 nm with FL1 photomultiplier. To establish an optimum condition for cell counting, a value of a discriminator was changed from 0 to 100. The stop time for the passage of the sample was set at 60 s, and an event number (number of particles passing through the laser beam) was measured as the number of yeast cells.

Conventional Method for Cell Counting

Colony counting method was used as shown below. Yeast cell suspension was diluted with 0.9% NaCl solution to obtain a final cell number of 30–300 cells per 100 μl. The cells were spread over the surface of YEPD agar plates. The plates were run in triplicate and were incubated for 48 h at 30°C. After incubation, the colonies on the plates were counted.

Results and Discussion

Effect of the value of the discriminator on the event number

Figure 1 shows a correlation between the event numbers (cells/60 s) and yeast cell concentrations for various values of the discriminator. A discriminator is a channel setting for a parameter allowing events below the setting to be ignored and it can eliminate signals caused by debris. The event number increased according to decreasing the discriminator on the low concentration of the cells. When the discriminator was set at 0, the event number did not change at all with the cell concentration and was represented as the straight line. This phenomenon may have been caused by the effects of the cell debris or the light scatter. On the other hand, when the discriminator was set at 15 or over, the event

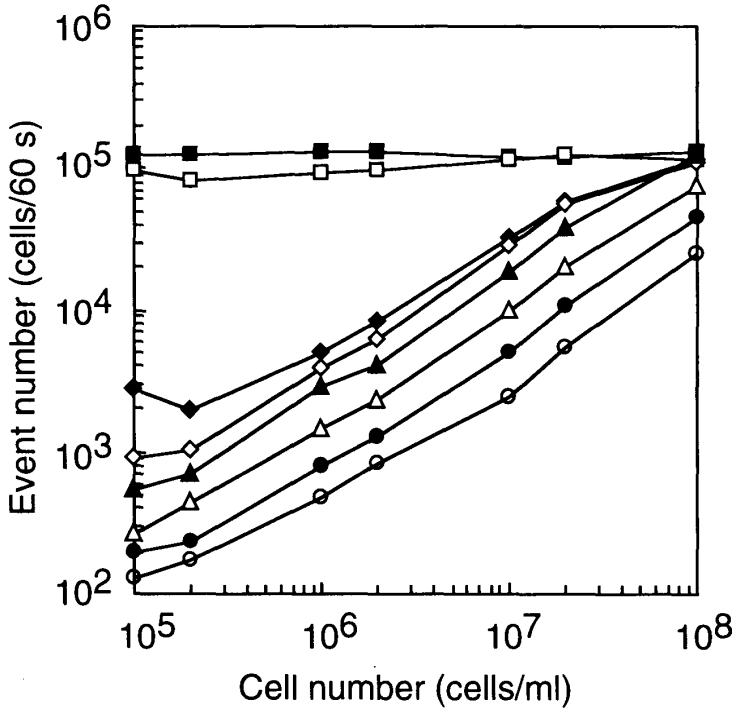

Fig. 1. The Effect of the discriminator on the event number of FCM.
The values of the discriminator were as follows: ■, 0; □, 10; ◆, 15; ◇, 20; ▲, 40; △, 60; ●, 80; ○, 100. A stop time of FCM was set at 60 s.

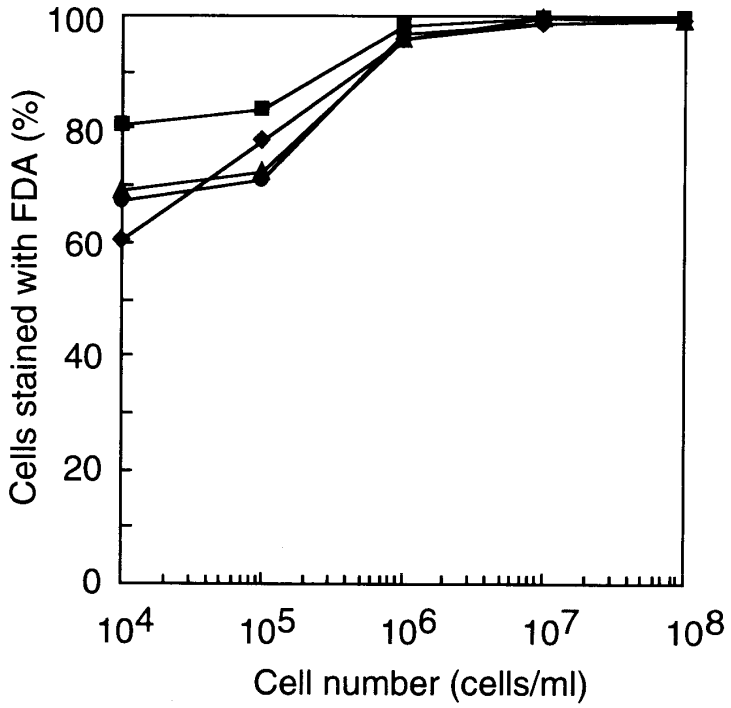

Fig. 2. The effect of FDA concentration on the number of stained cells.
The concentrations ($\mu g/ml$) of FDA were as follows: ▲, 1.0; ◆, 5.0; ■, 10.0; ●, 20.0. The conditions for the FCM assay were as follows; discriminate value, 60; stop time, 60 s.

number increased with increasing the cell concentration. A linear correlation was observed between the both parameters when the discriminator was set at 60. Therefore, all subsequent experiments were performed at this value.

Optimum concentration of FDA for stained cells

Figure 2 shows the effect of FDA concentration on the number of stained cells in various concentration of viable yeast cells. When the concentration of FDA was 1, 5, 10, or 20 $\mu g/ml$, the number of cells stained with FDA increased with increasing the cell concentration. The phenomenon could be assumed that apparently fraction of stained cells increased due to the aggregation of yeast cells by increasing cell concentration. At a FDA concentration of 10 $\mu g/ml$, the fraction of stained cells changed little with the cell concentration and above 80% of the cells were stained. For this reason, in subsequent experiments, the concentration of FDA was adjusted to 10 $\mu g/ml$.

The Distinction between Viable and Dead Cells with FDA

Figure 3 shows the fluorescence scattergrams (3.1a and 3.1b) and histograms (3.2a and 3.2b) of yeast cells stained with FDA.

In Figs. 3.1a and 3.1b, the horizontal axis represents FDA fluorescence intensity and the forward scatter intensity is plotted along the vertical axis. Increasing fluorescence intensity is represented by increasing channel number on the horizontal axis. The viable cells were stained by FDA and measured by the FCM (Fig. 3. 1a). Most of the cells were stained by FDA. Figure 3.1b shows the scattergram for the dead cells as a control. The stained cells were low rather than viable cells. From these results, it was assumed that FDA passed into the viable cell through the cell membrane and was retained inside of cells with an intact membrane. It is hydrolyzed by the cytoplasmic esterases of living cells and the resulting compound fluoresces green. In the case of dead cells, FDA leaked out of the cell which

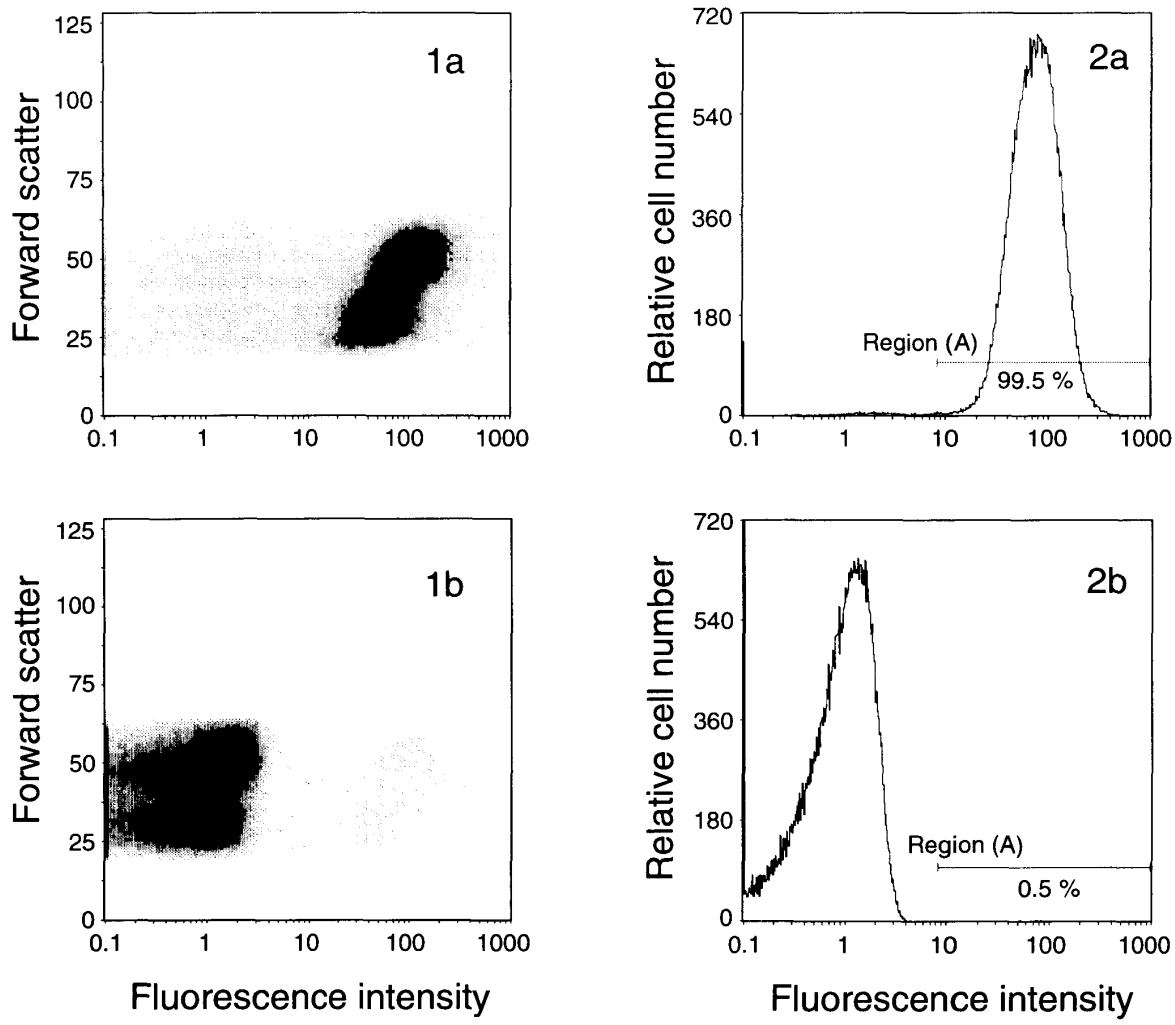

Fig. 3. Fluorescence scattergrams and histograms for yeast cells stained with FDA.
1, scattergram for FDA fluorescence intensity and the forward scatter intensity; 2, histogram for FDA fluorescence intensity and relative cell number.
a, viable cells; b, dead cells.
FDA concentration was 1 μg/ml. The FCM assay conditions were same as in Fig. 2.

consequently did not fluoresce.

Figures 3.2a and 3.2b show the fluorescence histograms for the same samples described above. The horizontal axis represents FDA fluorescence intensity and the vertical axis represents relative cell number. A linear region (A) was drawn in the histogram and was adjusted to distinguish between the viable cells and non-viable particles, that is, the particles within region (A) were regarded as the viable cells, and outside of (A) were considered the dead cells. When the viable cells were measured by the FCM, the ratio of viable cells in the region (A) was 99.5% (Fig. 3.2a). In the case of dead cells, only 0.5% of cells was contain in region (A) (Fig. 3.2b). Thus it is possible to discriminate between the viable and the dead cells using FDA staining.

Determination of the number of viable yeast by FCM

1) Calibration curve

Figure 4 shows the relationship between the event number of FCM and the cell number

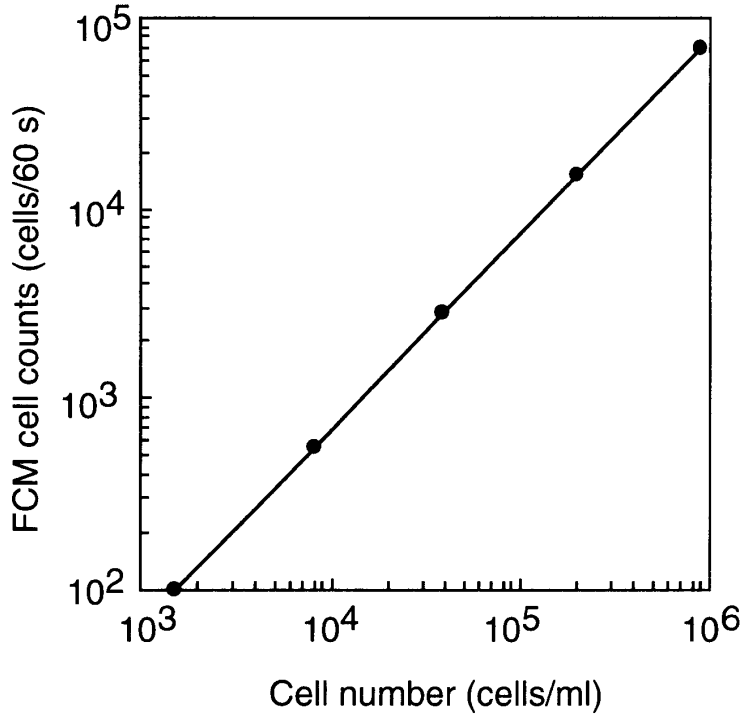

Fig. 4. Calibration curve for yeast cells.
The FCM assay conditions were same as in Fig. 2. FDA concentration was 1 μg/m*l*.

determined by the colony counting method. The calibration curve was linear in the range of 1.5×10^3 -9.0×10^5 cells/m*l*. Although the measurement of cell number less than 10^3 cells/m*l* was difficult, it was possible to determine low concentrations by increasing the sample volume. For example, if 1 m*l* of sample containing 10^3 yeast cells was passed through the cytometer at 100 μl/min, then it would be possible to detect 1,000 events in 10 min.

2) Application to fermentation process

FCM was applied for the determination of the cell number in fermentation process. Figure 5 shows the time cause of the event number of FCM, the cell number determined by the colony counting method, DO, and pH during fermentation, respectively. As shown in the figure, the dissolved oxygen concentration decreased with increasing the cell number. This phenomenon have been caused by the respiration activity of the microorganism. The event number of FCM increased corresponding to the cell growth and the logarithmic growth phase was also visible from these curves. When the number of dead cells was also determined by FCM, very few cells were contained in broth during fermentation (data not shown). The correlation between the proposed FCM method and the colony counting method was investigated in Fig. 6. The cell number was calculated from the calibration curves shown in Fig. 4. Linear relationships were observed in the range of 10^5–10^8 cells/m*l*. One FCM assay could be completed within 60 s and the total assay time including the preparation of microorganism was within 30 min.

In conclusion, our proposed method using FCM required significantly less time than the colony counting method and it could be used for the rapid determination of the number of viable yeast cells.

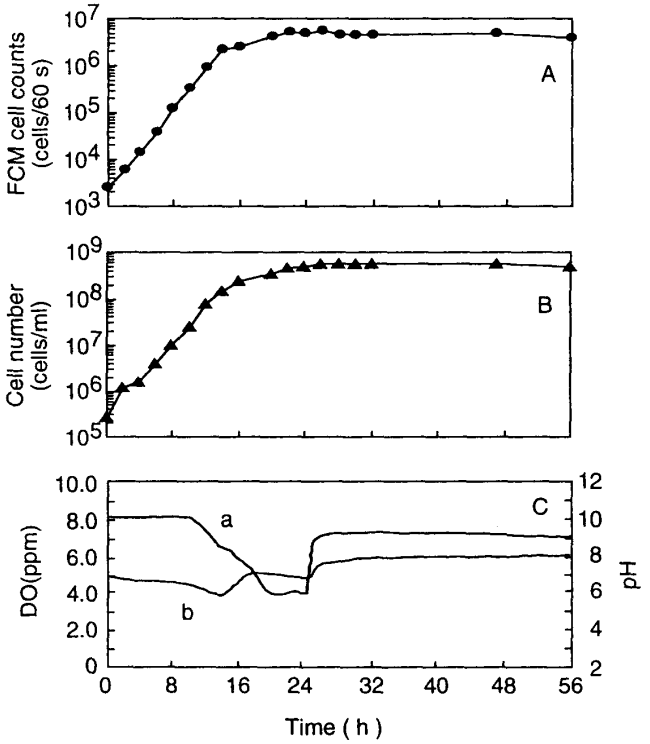

Fig. 5. Time course of the event number of FCM, the cell number, DO, and pH during fermentation. A, FCM cell counts; B, cell number determined by the colony counting method; C, DO and pH. The FCM assay conditions were same as in Fig. 2. FDA concentration was 1 μg/ml.

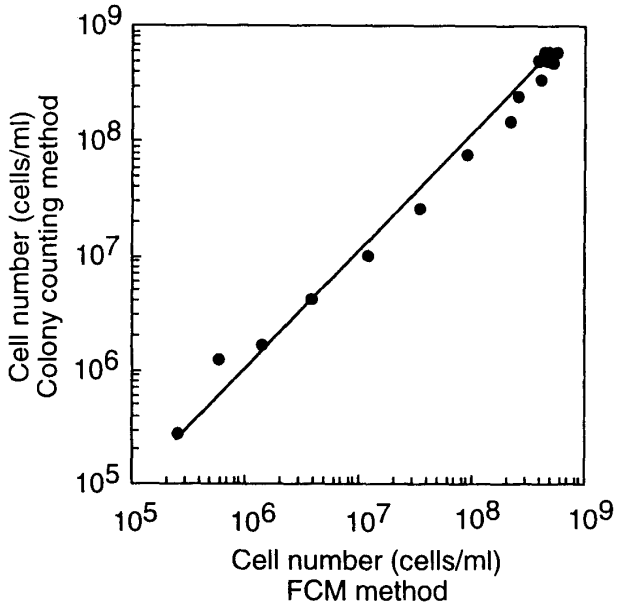

Fig. 6. Correlation between the proposed FCM method and the colony counting method. The FCM assay conditions were same as in Fig. 2. FDA concentration was 1 μg/ml.

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フローサイトメトリーによる酵母生菌数の迅速測定

遠藤英明・林　哲仁・中山純子・向田喜昭・渡辺悦生

フローサイトメトリー (FCM) を応用し，培養下酵母の迅速な生菌数測定を試みた。まず Fluorescein diacetate (FDA) を用いて，酵母の生細胞と死細胞を染色し，FCM を用いて解析した。その結果，染色と非染色細胞のスキャッタグラムには顕著な差が認められ，それらの識別が可能であった。このときの FDA の染色濃度は 10 g/ml が最適であった。次に FCM を用いて培養下酵母の生菌数計測を試みたところ，FCM のイベント数と酵母の増殖曲線との間には同様の経時変化が認められた。そして検量線を用いて生菌数を算出したところ，10^5～10^8 cells/ml の範囲で平板培養法で得られた結果との間に良い相関関係が認めらた。さらに一検体の測定所要時間は 60 秒であり，菌体の前処理を含めても 30 分以内で測定が可能であった。

キーワード: フローサイトメトリー，酵母，菌数，Fluorescein diacetate