MEASUREMENT OF VIABLE ANIMAL CELL CONCENTRATION BASED ON FLUOROMETRY

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In viable animal cells, fluorescein diacetate is hydrolyzed to fluorescein (a fluorescent compound) which is retained in the cells. Fluorescein was effectively extracted from the cells by treatment with hypotonic phosphate buffer ($1 \text{ mmol} \cdot \text{dm}^{-3}$, pH 7.4) and the extract showed a fluorescent peak at excitation and emission wavelengths of 496.5 and 515 nm, respectively. A linear relationship was confirmed between the viable cell concentrations based on fluorometry and microscopy. Animal cell growth could be successfully monitored based on fluorometry for suspension and microcarrier cultures of animal cells.

Introduction

In vitro culture of animal cells has become increasingly important for the production of economically valuable substances such as vaccines and monoclonal antibodies, which are not usually synthesized by microbial cells. In the last decade, many studies of animal cell culture systems have been intensively performed, including suspension, microcarrier and immobilized-cell cultures.^{1-5,16} In animal cell culture, it is extremely important to estimate viable cell concentration because desired products are usually formed according to viable cell level. To date, the microscopic counting of stained cells with trypan blue, erythrosine B and so on has been used exclusively for estimation of viable cell concentration.¹⁵

However, this method often lacks the desired accuracy or reproducibility at low cell concentration, and requires time and labor-consuming procedures such as detaching cells from microcarriers by trypsinization.

In previous papers,^{13,14} we reported the application of fluorometry to microbial cultures and demonstrated that it offered significant information on microbial growth and activity. In the present study, we measure concentration of viable mammalian cells on the basis of fluorometry. Fluorescein diacetate (FDA) has been used to determine the viability of plant cells or protoplasts.^{7,17} As shown in **Fig. 1**, non-fluorescent FDA molecules pass freely across the plasma membrane of a cell and are hydrolyzed by esterases in the cell. This reaction results in the formation of fluorescein (FRC), a fluorescent compound. FRC is retained within the living cell having

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Fig. 1. Behavior of FDA and FRC molecules in viable and nonviable cells

an intact membrane, as shown in Fig. 1, while it diffuses out across the membrane in a non-viable cell. This is due to permeability difference through the membrane between nonpolar (FDA) and polar (FRC) compounds.¹²⁾ Thus, FDA is a fluorogenic substrate and can be used as a labeling reagent for living eucaryotes. This paper describes the quantitative analysis of viable animal cells on the basis of fluorometry.

1. Experimental

1.1 Cell lines and media

The cell lines used in suspension cultures were mouse hybridoma 16-3F cell⁶⁾ (producer of monoclonal antibody against recombinant α -amylase) and RPMI 8226 human hematopoietic cell.⁹⁾ For microcarrier culture, rabbit kidney cell, RK₁₃ (from the American Type Culture Collection, No. ATCC CCL 37) was used. The 16-3F and RPMI 8226 cells were cultivated in the medium with fetal calf serum (FCS) (NS-1 medium) or without FCS (DF-ITES medium) as reported previously.^{6,15)} The RK₁₃ cells were grown in Dalbeco-modified Eagle (DME) medium¹¹⁾ supplemented with 10% (v/v) FCS.

1.2 Culture conditions

All cultivations were performed at 37°C in a humidified 5% (v/v) CO₂ atmosphere using 10-cm petri-dishes (Corning Glass Co.) containing 10 to 20 cm³ of medium. The microcarrier culture of RK₁₃ cells was carried out according to the procedures reported by the supplier,¹¹⁾ and Cytodex 1 was used as a microcarrier.

1.3 Fluorometric measurement and other analyses

Throughout the experiments, a Model FP-770 spectrofluorometer (Japan Spectroscopic Co.) was used. The procedure of fluorescence measurement is briefly described in Fig. 2. The cells (or microcarriers with cells) were washed with PBS solution (NaCl: 8 kg; Na₂HPO₄ · 12H₂O: 2.9 kg; KH₂PO₄: 0.2 kg; and KCl: 0.2 kg per m³, pH 7.4) and resuspended in the solution. The suspension aliquot was mixed with one volume of FDA solution (96 μ mol per dm³ of PBS). The cells were washed with PBS and subjected to cell disruption such as sonication (five times at

Fig. 2. Procedure of cell treatment for fluorescence measurement

0.5 min intervals at 60 W using an Ohtake Works Type 5202 cell disrupter) or solvent treatments. The resultant fluorescence intensity was measured mainly at *Ex* of 496.5 nm and *Em* of 515 nm after cell debris removal by centrifugation. Authentic fluorescein Na solution (0.026 to 2.6 μ mol · dm⁻³) was used for standardization of measurement conditions, and the fluorescence intensity was expressed as FRC concentration.

As a conventional method, viable cell concentration was determined by microscopic counting with a haemocytometer according to the dye exclusion test.¹⁵⁾ At low cell concentration, the cells were counted after concentrating them by centrifugation and resuspending them in PBS solution. Glucose concentration was measured with a glucose analyzer (Model 27, Yellow Springs Instrument Co.).

1.4 Chemicals

Fluorescein · Na and FDA were purchased from Wako Pure Chemical Industries and Aldrich Chemical Co., respectively. Cytodex 1 was commercially available from Pharmacia Fine Chemicals. The medium components were described elsewhere.^{6,15)} The other chemicals used were of analytical grade.

2. Results and Discussion

2.1 Conditions for fluorometric measurement

Following the procedure shown in Fig. 2, cell

extract of 16-3F cells was prepared after mixing with FDA (cell disruption: sonication). **Figure 3** indicates the fluorescence spectra of the extract, authentic FRC solution and so on. The *Ex* was predetermined as 496.5 nm, so as to give the highest fluorescence intensities against these solutions. The fluorescent peak of the cell extract with FDA completely coincided with that of FRC with a maximum at 515 nm, while no significant fluorescence was observed for the cell extract without FDA. Thus, it was confirmed that FDA converted to FRC in the animal cells, and that the cell extract exhibited sufficient fluorescence intensity.

Figure 4 shows the effect of incubation time for the hydrolysis of FDA on fluorescence intensity. Some reaction time may be necessary for complete hydrolysis of FDA in the cells. However, no incubation time was necessary, as shown in Fig. 4, since a few minutes were required for the centrifugation after addition of FDA. This result suggests that the cell's capacity for FDA (and/or FRC) was limited and the cells were immediately saturated with these compounds.

To achieve simple but efficient cell disruption, various methods were next examined such as sonication (see "Experimental") and suspending the cells in solutions (detergents, organic solvents, buffers etc.) after cell harvest by centrifugation (see Fig. 2). **Table 1** shows the effects of the treatments on the fluorescence intensity released from the cells. In the case of hypotonic solution treatments ($1 \text{ mmol} \cdot \text{dm}^{-3}$ phosphate and Tris-HCl buffers, pH 7.4) where cell burst was microscopically confirmed, the resultant extracts exhibited fluorescence comparable to that from the sonication. In the case of Tris-HCl buffer, however, the treatment efficiency declined for the sample with high cell concentration (data asterisked in Table 1). Unusual results were obtained in NaOH solution and water treatments which may be attributable to pH values (pH of water: 6 to 6.5).

Figure 5 shows the fluorescence intensities in hypotonic phosphate buffers of various pH values. The fluorescence fell sharply below pH 7. This phenomenon seems to result from the quenching of FRC fluorescence in acidic solution.¹⁸⁾ On the other hand, non-enzymatic cleavage of FDA was observed in high pH regions, leading to enhanced fluorescence intensity at higher pH value. Thus, the phosphate buffer of pH value around 7.5 was appropriate as a hypotonic solution.

Based on the results above, subsequent experiments were done as shown in Fig. 2 under the following conditions: (A) no intended incubation time after FDA addition; (B) cell disruption and FRC release in hypotonic phosphate buffer ($1 \text{ mmol} \cdot \text{dm}^{-3}$, pH 7.4); and (C) *Ex*: 496.5 nm and *Em*: 515 nm.

Fig. 3. Fluorescence spectra of cell extracts, and authentic FDA and FRC (*Ex*: 496.5 nm). The cell extracts of 16-3F cells were prepared by sonication as shown in Fig. 2

Fig. 4. Effect of incubation time after FDA addition on fluorescence intensity. The 16-3F cells ($7.5 \times 10^5 \text{ cell} \cdot \text{cm}^{-3}$) were mixed with FDA, incubated at 37°C for a given time and treated by sonication. Fluorescence intensity was measured at *Ex* = 496.5 and *Em* = 515 nm.

Table 1. Effects of various treatments on FRC extraction from cells

Treatment	Relative F [—]
Sonication	1.0
SDS ($140 \text{ mmol} \cdot \text{dm}^{-3}$)	0.07
Triton X100 ($15 \text{ mmol} \cdot \text{dm}^{-3}$)	0.33
Brij 35 ($8.3 \text{ mmol} \cdot \text{dm}^{-3}$)	0.01
Acetone ($14 \text{ mol} \cdot \text{dm}^{-3}$)	0
Ethanol ($17 \text{ mol} \cdot \text{dm}^{-3}$)	0
NaOH ($100 \text{ mmol} \cdot \text{dm}^{-3}$)	2.1
NaOH ($10 \text{ mmol} \cdot \text{dm}^{-3}$)	2.1
NaCl ($30 \text{ mmol} \cdot \text{dm}^{-3}$)	0.39
Phosphate buffer ($10 \text{ mmol} \cdot \text{dm}^{-3}$, pH 7.4)	0.47
Phosphate buffer ($1 \text{ mmol} \cdot \text{dm}^{-3}$, pH 7.4)	1.1, 1.1*
Tris-HCl buffer ($1 \text{ mmol} \cdot \text{dm}^{-3}$, pH 7.4)	1.3, 0.49*
Water	0.43
No treatment	0.02

The 16-3F cells ($3.0 \times 10^5 \text{ cell} \cdot \text{cm}^{-3}$) were subjected to the treatments shown in Fig. 2. Figures with asterisk are the results for high cell concentration ($1.2 \times 10^6 \text{ cell} \cdot \text{dm}^{-3}$). *Ex*: 496.5 nm; *Em*: 515 nm.

Fig. 5. Fluorescence intensity of cell extract and FDA in hypotonic phosphate buffers ($1 \text{ mmol} \cdot \text{dm}^{-3}$) of various pH values. Fluorescence intensity was measured at $E_x=496.5$ and $E_m=515 \text{ nm}$ after FDA was added to the 16-3F cells ($9.7 \times 10^5 \text{ cell} \cdot \text{cm}^{-3}$) and PBS (as a control), followed by treatment with the hypotonic phosphate buffers.

2.2 Relationship between fluorescence intensity and viable cell concentration

The viable cell concentration numerated by microscopy is plotted against the fluorescence intensity derived from the cells in Figs. 6 (for 16-3F cells) and 7 (for RK₁₃ cells). In the latter case, the viable cell number was counted by microscopy after cell detachment from the microcarrier by trypsinization,¹¹⁾ while the fluorescence intensity was determined without detachment (Fig. 2). For 16-3F and RK₁₃ cells, straight lines were obtained with the slopes of 4.5 and 5.0×10^{-7} , respectively, which indicate constant FRC content per cell. As shown in the inset in Fig. 6, good linearity was also observed for the low-cell density range, where the viable cell number on the abscissa was determined after adequate concentration of the cells.

Table 2 summarizes the FRC content of some animal cells. For the cells cultivated in the media with FCS, the content values ranged from 0.32 to $0.50 \text{ fmol-FRC} \cdot (\text{cell})^{-1}$. In the investigation with 16-3F cells, the cells in the FCS-free medium showed higher FRC content than those in the FCS medium. Though the reason is not obvious, the cells may have a little larger cell size or altered membrane permeability against FDA.

2.3 Estimation of viable cells during cultures by fluorometry

Using the FRC content values in Table 2, it is possible to estimate viable cell concentration in a given culture on the basis of the fluorometric measurement. Figure 8 shows the suspension culture of 16-3F cells using the DF-ITES medium without FCS. The viable cell concentration on the fluorometric basis was approximately in agreement with that on

Fig. 6. Relationship between fluorescence intensity and viable cell concentration counted by microscopy for 16-3F cells. The cells were cultivated in suspension with the NS-1 medium containing FCS. The inset shows the data for low cell concentration.

Fig. 7. Relationship between fluorescence intensity and viable cell concentration counted by microscopy for RK₁₃ cells. The cells were cultivated on microcarriers with DME medium containing FCS

Table 2. Values of FRC content per cell for various cells

Cells	Culture medium	F/X [fmol-FRC · (cell) ⁻¹]
16-3F	NS-1 with FCS	0.45 (0.034)
16-3F	DF-ITES without FCS	0.63 (0.034)
RK ₁₃	DME with FCS	0.50 (0.038)
RPMI 8226	NS-1 with FCS	0.32 (0.050)

Figures in parentheses are standard deviations.

the microscopic basis, although a small discrepancy was observed in the early growth phase. The microcarrier culture of RK₁₃ cells was also performed, where the microcarriers were transferred to fresh DME medium with FCS several times. As shown in Fig. 9, the fluorometrically determined cell numbers coincided well with the microscopic ones.

Recently, some workers reported application of fluorometry to animal cell cultures. MacMichael *et al.*⁸⁾ demonstrated the quantitative determination of viable hybridoma cell concentration by the measurement of fluorescent coenzyme, NAD(P)H. Using an

Fig. 8. Time course of 16-3F cell suspension culture. The cells were cultivated with FCS-free DF-ITES medium

Fig. 9. Time course of RK₁₃ cell microcarrier culture. The cells were cultivated with DME medium containing FCS and were transferred to fresh medium at the time indicated by the arrows

FDA derivative, moreover, Margaret *et al.*¹⁰⁾ performed the fluorometric approach to physiological analysis of communication between contiguous human cells mediated by gap junction (transport channel for low-molecular weight cytoplasmic components). In the present work, we proposed the quantitative estimation method of viable animal cells on the basis of fluorometry with the FDA-FRC system. This method was applicable to a relatively wide range of cell concentration without a prior preparation of the cell suspension such as condensation or dilution operation. Although FRC extraction from cells by hy-

potonic solution treatment was necessary before the fluorescence measurement, this step was neither troublesome nor time-consuming. Therefore, the method was convenient for estimation of support-anchored cells because of the absence of a cell-release step.

Conclusion

1) The FDA molecule was hydrolyzed to the fluorescent compound FRC in viable animal cells and the FRC was retained in the cells.

2) FRC molecules were effectively extracted from the viable cells by suspending the cells in hypotonic solution (1 mmol·dm⁻³ phosphate buffer, pH 7.4). The extract exhibited the highest fluorescence intensity at $Ex=496.5$ and $Em=515$ nm.

3) A linear relationship was observed between the viable cell concentrations based on fluorometric determination and microscopic counting for a wide range of cell concentration (order of 10⁴ to 10⁶ cell·cm⁻³).

4) The FRC content per cell was determined for several animal cells. Using the content value, it was possible to estimate the viable cell concentration for suspension and microcarrier cultures of the animal cells on the basis of fluorometry.

Nomenclature and Abbreviations

Em	= emission wavelength	[nm]
Ex	= excitation wavelength	[nm]
F	= fluorescence intensity	[nmol-FRC·cm ⁻³]
FCS	= fetal calf serum	
FDA	= fluorescein diacetate	
FRC	= fluorescein	
G	= glucose concentration	[μmol·cm ⁻³]
t	= cultivation time	[d]
X	= viable cell concentration	[cell·cm ⁻³]
NAD(P)H	= nicotinamide-adenine dinucleotide (phosphate)	
PBS	= phosphate-buffered saline	
SDS	= sodium dodecyl sulfate	

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