A rapid method for evaluation of cell number and viability by flow cytometry

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Abstract

A simple, rapid and reliable method has been developed for assessing the number and viability of cells, as well as cell size, in suspension culture by the use of flow cytometry. Propidium iodide exclusion is used for viability determination and fluorescent beads serve as an internal standard for cell enumeration. The main advantages of this method are its ability to handle a large number of samples with a high degree of precision and its specificity in detecting viable cells quantitatively in a heterogeneous culture of living and dead cells and debris. The method shows only a fraction of the variation found in the haemocytometer/trypan blue counting method due to its very low operator dependence.

Abbreviations: CHO – Chinese hamster ovary; FCS – Foetal calf serum; FS – Forward scatter light; MTT – 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NCS – newborn calf serum; PBS – Phosphate buffered saline; PI – Propidium iodide; SS – Side scatter light.

Introduction

The economic importance of protein production by *in vitro* cultured animal cells has greatly increased in recent years. Along with product yield, one of the fundamental parameters of a cell culture process which must be accurately monitored is the change in cell density i.e. the growth of the culture. This is not only because reliable growth measurement is important for monitoring the quality and course of a bioprocess but also because culture parameters such as growth rate, specific productivity, specific metabolic quotients and yields are derived from the growth measurement.

The quantifiable distinction between proliferative behaviour and cell death is fundamental to bioprocess characterisation. Classical methods for assessing viability and cell number, e.g. the haemocytometer/trypan blue exclusion method, are labour intensive and slow when handling large numbers of cultures. Counting cells with a haemocytometer is regarded as being sub-

ject to inter-observer variation. Reasons for such variation may include; counting too few squares and too few cells; uneven distribution of cells in the sample or in the haemocytometer chambers (sampling error); incorrect dilution; overfilling or variation of haemocytometer filling rate (Nielsen *et al.*, 1991). A recent modification to the traditional method, using automated image analysis, addressed some of the problems of such inter-observer variation and the subjective discrimination between small cells and large debris (Tucker *et al.*, 1994). However this method is still relatively time consuming, taking approximately 22 minutes to analyse one sample. It has to be noted that precise viability assessment by membrane integrity examination is always difficult, as one has to take into account the fact that cells with little membrane damage may still be viable and have the potential for repair and further proliferation.

Another method for counting cultured cells is to use a Coulter particle counter. This rapid method has the

Figure 1. Typical gates for enumeration of CHO320 and TB/C3 hybridoma cells: gate A: beads, gate B: viable cells, gate C: viable and dead cells, gate D: viable cells, gate E: dead cells. FS = forward scatter, PI = propidium iodide.

advantage that cells can at least be distinguished objectively from debris on the basis of size, thus overcoming one problem of interobserver variation. Furthermore a large number of particles can easily be counted, thus reducing sampling error. However, a major disadvantage of this method is that there is no discrimination possible between viable and dead cells.

Alternative assays for characterising the state of a culture include measurement of DNA, RNA and protein contents, or their rates of synthesis. Other measurements of metabolic activities used as indicators of cell growth include; the MTT test, lactate dehydrogenase release and the ^{51}Cr release test (Mosmann, 1983; Al-Rubeai and Spier, 1989; Cook and Mitchell, 1989; Goergen *et al.*, 1993). These alternatives have been extensively reviewed elsewhere (Wilson, 1986; Capiaumont *et al.*, 1993; Henkes *et al.*, 1993).

Viability assessment always leads to the problem of defining what is a viable cell. Presumably, the best method for bioprocess-related definition is to relate the viability of a culture to the number of cells in it with reproductive potential and to carry out cloning efficiency tests ("clonogenic assays"). These methods are however very time consuming and entirely unsuitable for routine process measurements of viability.

In this paper we describe a new flow cytometric method for counting animal cell suspension cultures. The method exhibits the advantages of speed, ability to handle large numbers of cells (reduced sampling error) and most particularly an objective discrimination between viable and dead cells as well as distinct exclusion of debris from the counting process.

Materials and methods

Cell culture

The mouse:mouse hybridoma cell line TB/C3 producing antibody against human IgG was grown in RPMI 1640 (Gibco) supplemented with 5% NCS (Sigma). A recombinant CHO 320 cell line producing human interferon- γ (kindly provided by GlaxoWellcome, Kent, UK) was grown in RPMI 1640 (Gibco) supplemented with 5% FCS (Advanced Protein Products) and 10 μM methotrexate (Sigma). 200 ml batch culture of both cell lines were grown at 37 °C in 500 ml bottles (Duran), stirred at 150 rpm. 5 ml samples were taken off-line for counting. CHO320 was used for all experiments except where stated otherwise.

Manual counting and viability assessment

Classical manual counting was done using an Improved Neubauer counting chamber and a binocular microscope at 200 $\times$ magnification. Trypan blue exclusion (0.25% final dye concentration) was used for viability assessment. At least 12 big squares and 3 fillings of the counting chamber were assessed by experienced researchers. For calibration experiments both sides of the haemocytometer were filled 5 times and all 18 squares were examined.

Counting and viability assessment by flow cytometry

500 μl cell suspension was added to 100 μl stock staining solution, mixed by gentle shaking and analysed immediately. The stock staining solution contained uniform fluorospheres (Immuno-Check, 10 μm , Coulter) as an internal standard ($1.1 \times 10^5 \text{ ml}^{-1}$) and PI

(250 $\mu\text{g ml}^{-1}$) in PBS, pH 7.2. The stock staining solution was stable for at least 3 weeks when stored at 4 °C in the dark (adequate DNA staining and no clumping of beads). Immediately after staining, flow cytometric analysis was performed using a Coulter EPICS Elite analyser equipped with an argon laser (488 nm). Red fluorescence (PI) was collected using a 635 nm band pass filter after blocking laser light with a 488 nm long pass filter. Analysis was undertaken by loading an appropriate protocol (see results) for the acquired parameters; FS, log SS and PI integral; either 10000 or 20000 observations were collected for analysis. An example analysis is shown in Figure 1. The evaluation of cell number was achieved by gating areas in the FS vs log SS dot plot in which beads, living cells and dead cells appear. Cell number ml^{-1} was calculated from the number of beads (gate A) in relation to the total number of cell shaped observations (gate B) using the following equation:

$$\text{Cell density (n ml}^{-1}\text{)} = \frac{\text{area gate B} \times \text{bead concentration in staining solution (n ml}^{-1}\text{)}}{\alpha \times \text{area gate A}}$$

where; n = number,

α = dilution factor of staining solution.

Cell death is known to be associated with a change in light scattering properties (Darzynkiewicz *et al.*, 1994). Therefore, to include spherical dead cells in the viability determination a further gate has to be set (gate C). For quantification of viability, the ratio of viable cells with little or no PI staining (gate D) to cells with a significant intensity of PI staining (gate E) i.e. dead cells, was calculated. Appropriate gates were set in the PI vs. FS plot. The same protocol was used for both hybridoma and CHO cells.

Results and discussion

Setting up a new protocol

Different cell lines show slightly different forward and side scatter properties. Therefore it is necessary to adjust the protocol for each cell line analysed. However, once established, the same protocol can be used for all cultures of the same cell line e.g. CHO, hybridoma *etc.* Initial empirical adjustment allows the best system and gate parameters to be achieved by accurately analysing two samples, one taken from the exponential growth phase and one from the late stationary phase.

Once this calibration has been made, analysis of cell number and viability can be reliably repeated.

Maximum data rate and cell density

To investigate the influence of the data collection rate on the counting results, two samples were analysed by varying the sample pressure. Figure 2 shows that cell counting is feasible up to a data rate of higher than 1500 (instrument specified rate). The highest data rate possible is dependant on the cell density of the sample. A CHO320 cell density from $1 \times 10^5 \text{ ml}^{-1}$ to a maximum of $3 \times 10^6 \text{ ml}^{-1}$ was reliably analysed without sample dilution (Figure 3). To measure higher cell densities, the sample should be diluted appropriately before analysis.

Reproducibility and objectivity

To demonstrate the suitability of this procedure for other cell lines, a batch of hybridoma TB/C3 was analysed.

Figure 4 shows that the flow cytometric method is also suitable for hybridoma cells. It is notable that there is good agreement between the two counting methods (manual and flow cytometric) in the later stationary phase when the heterogeneity of the suspension increases.

Precision and operator dependence

Table 1a shows the precision of the flow cytometric method and the influence of the operator on counting and viability measurements. Individual variation resulted in a coefficient of variation of 5.3% even between the counts of 6 different operators. Replicate analysis by one operator gave a coefficient of variation of only 1.4%. The respective values for viability determination were 0.64% and 0.15%.

Flow cytometry compared with traditional method

a. Evaluation of cell number

Figure 5 and Table 1b show that the cell density determined by the manual method is subject to a high degree of variation; up to 20% compared with just over 5% for the flow cytometry method. The method is also subject

Figure 2. Influence of the data rate on flow cytometer counts: Analysis of two CHO 320 cell samples under different speeds of sample flow; ▲ cell density sample 1, ▼ cell density sample 2, ● viability sample 1, ■ viability sample 2.

Figure 3. Estimation of the highest CHO320 cell density measurement possible by flow cytometric analysis (data rate 200–800).

Figure 4. TB/C3 hybridoma cells during batch culture; ▼ cell density by flow cytometry, ○ manual cell density determination, ▲ viability by flow cytometry, □ manual viability determination.

Figure 5. Comparison of CHO320 cell density and viability determined manually by different operators (n=5) and the flow cytometric method; ▼ cell density by flow cytometry, ▲ viability by flow cytometry, ○ manual cell density determination, □ manual viability determination.

Table 1. a) Operator influence on flow cytometric analysis of CHO320 cells, b) Statistical evaluation of a trypan blue stained manual cell counting experiment conducted by 5 operators examining CHO cells

a)								
	Different operators				Same operator			
	Cell density ($\times 10^5 \text{ ml}^{-1}$)		Viability (%)		Cell density ($\times 10^5 \text{ ml}^{-1}$)		Viability (%)	
min	5.70		97.8		5.77		99.0	
max	6.39		99.2		5.98		99.4	
mean	6.09		98.6		5.89		99.2	
observations, n=	6		6		7		7	
standard deviation	0.321		0.643		0.085		0.152	
coefficient of variation (1%)	5.3		0.64		1.4		0.15	
b)								
Incubation (h)	14	21	38	44	58	82	108	114
Cell density ($\times 10^5 = \text{cells ml}^{-1}$)								
min	1.69	2.22	4.16	4.91	6.42	10.19	12.40	10.32
max	2.50	3.70	4.77	6.08	9.15	13.38	14.16	12.53
mean	2.17	3.03	4.48	5.53	7.51	11.27	13.46	11.50
operators, n=	5	5	5	5	5	5	5	2
standard deviation	0.311	0.609	0.265	0.508	1.278	1.285	1.625	1.330
coefficient of variation (%)	14.3	20.1	5.9	9.2	17.0	11.4	12.1	11.6
Viability (%)								
min	89.5	90.0	94.4	92.0	93.7	95.6	93.6	92.2
max	99.2	97.5	97.8	98.0	98.2	98.2	94.7	95.0
mean	94.0	95.1	96.0	95.4	95.6	97.4	94.1	93.2
operators, n=	5	5	5	5	5	5	5	2
standard deviation	3.7	3.0	1.7	2.3	1.9	1.1	0.5	0.1
coefficient of variation (%)	4.0	3.1	1.8	2.4	2.0	1.1	0.6	0.1

tive and operator dependent. Differences can occur due to individual pipetting technique, non homogeneous distribution of cells, by including cell fragments in the count, and the individual's use of the counting chamber. Furthermore, only a small fraction of the cells per sample volume is counted. This means that multiplication factors of 2000–50000 are used which exaggerate the errors described above. Nielsen *et al.* (1991) reported that a maximum precision of 22% is reached when all 9 squares and both sides of one haemocytometer are counted. Cell density measurement reach precisions of 16% and 13 % for two and three fillings respec-

tively. The questionable comparability of experiments carried out by different operators strengthens the need for a reproducible operator-independent automation.

b. Assessment of viability

Manually determined viability is also less precise than measurement based on flow cytometry. The first sample shown in Figures 5 and Table 1b gave a coefficient of variation of 4% when using the traditional method, compared with one of less than 1% for the flow cytometry method. The difference in viability decreases obviously when more cells are analysed. The most like-

Figure 6. 3-D plot (FS vs. Log SS) showing beads and viable cells as distinct populations. Sample from batch culture of CHO320 cells after 32 h incubation.

ly reason why viability by the trypan blue exclusion method is assessed with such a high degree of variation is that, as in the evaluation of cell number, different operators include different proportions of blue stained debris in their calculations.

c. Speed of analysis

Manual assessment of cell number and viability for one sample takes approximately 15 min. This depends on the number of cells present in the sample, the number of squares counted, the number of fillings of the counting chamber and the experience of the operator. In comparison, the flow cytometric measurement was only two pipetting steps followed by the flow cytometric analysis and provided that the flow cytometer is calibrated, a typical analysis takes about 1 min.

Potential problems, solutions and restrictions

In the later stages of a batch culture, increased amounts of debris are to be found. For counting by flow cytometry it is essential that the beads from a distinct population in the FS vs. log SS dot plot (Figure 6). If this is not the case and debris appears in the bead gate, the number of cells will be under-estimated. This problem can be overcome by using highly uniform beads and/or different sized beads and/or altering the sensitivity of the FS and SS photomultipliers.

Some cell lines, including CHO cells, may aggregate, especially in the later phases of a batch culture. This may equally affect both the microscopic and the flow cytometric count. Current methods to overcome this problem include repeated aspiration up

and down hypodermic needles, addition of trypsin or addition of DNase. However, the effect of these treatments on trypan blue or PI uptake and hence their influence on viability assessment is not known – nor indeed considered in the literature. Experience suggests that repeated aspiration up and down hypodermic needles may detrimentally affect cell viability.

For routine measurements in animal culture processes, cells are considered to be viable as long as they do not take up membrane impermeable stains such as PI. However, PI can be taken up by intact cells after prolonged exposure. This problem is also encountered with trypan blue staining. However, the immediacy of flow cytometric analysis, using only 20 s staining time overcomes this problem. PI may also bind non-specifically to cell surface proteins. If this problem occurs, other more specific DNA stains such as ethidium homodimer 1 (Molecular Probes) might be used instead of PI following a similar experimental protocol.

Conclusion

Results presented in this paper show that flow cytometry can be applied as a rapid, precise and objective tool for routine measurement of cell density and viability in animal cell suspension cultures. The method addresses and overcomes the problems of subjectivity, and some of the inherent sampling errors associated with using the traditional haemocytometer and trypan blue exclusion method. Furthermore, since the only

manual steps required are sample aspiration, mixing with stain/beads and making 2 simple calculations, it is feasible that, with further development, the whole method could be automated and brought on line for process monitoring and control.

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