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RESEARCH ARTICLE

NucleoCounter—An efficient technique for the determination of cell number and viability in animal cell culture processes

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Abstract The NucleoCounter is a novel, portable cell counting device based on the principle of fluorescence microscopy. The present work establishes its use with animal cells and checks its reliability, consistency and accuracy in comparison with other cytometric techniques. The main advantages of this technique are its ability to handle a large number of samples with a high degree of precision and its simplicity and specificity in detecting viable cells quantitatively in a heterogeneous culture. The work 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. NucleoCounter offers reduced intra- and inter-observer variation as well as consistency in repetitive analysis that establishes it as an efficient and highly potential device for at-line monitoring of animal cell processes. Furthermore, since the only manual steps required are sample aspiration and mixing with two reagents, it is feasible that the whole method could be automated and brought on-line for process monitoring and control.

Keywords NucleoCounter · Cell culture · Monitoring · Flow cytometry · Cell count

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Introduction

Trypan Blue/Haemocytometer is the conventional method for counting cells. This method has been used widely in industry, as it is cheap, reliable and easy but it has major shortcomings due to subjective determination of cell count, as well as involving manual and time consuming steps. This operation results in large inter-user variability (Nielson et al. 1991). The reasons for this might be counting too few squares, uneven distribution of cells (sampling errors), incorrect dilution, overfilling and variation of haemocytometer filling rate (Al-Rubeai et al. 1997). There might also be an error, if using different pipette.

Alternative techniques for rapid cell counting include flow cytometry-based devices which measure the fluorescence emission of cells or nuclei in suspension. This rapid method has the advantage that viable and dead cells can at least be distinguished objectively from debris on the basis of size and granularity, thus overcoming one problem of inter-observer variation. Furthermore a large number of particles can easily be counted,

thus reducing sampling error and producing data with higher statistical validity (Al-Rubeai et al. 1997; Harding et al. 2000; Mukwena et al. 2003).

Cell number can also be counted in several other systems including the cell counter and analyzer system CASY 1 (Schärfe System, Reutlingen, Germany). Measurements in this system are based on detection of conductivity changes along an aperture during the flow of cell-containing liquid. The result is a cell size distribution curve in which cell debris, dead cells and viable cells can be identified by their distinct diameters (Falkenhain et al. 1998). Dead cells are characterized by the volume of their cell nucleus which alone contributes to the conductivity (Winkelmeier et al. 1993). Coulter counter (Multisizer 3 CoulterCounter, Beckman Coulter, Inc., USA) is used to provide number, volume, mass and surface area size distributions. The system is capable of counting particles at overall sizing range of 0.4 µm–1,200 µm. Recently, an integrated analysis system for automatic cell density and viability determination (Cedex®) based on optical recognition has been developed to measure cell density and viability. Kwok et al. (2002) have shown a good correlation between trypan blue and Cedex viable cell counts in 3 hybridoma cell lines.

NucleoCounter is a novel technique based on the fluorescence microscopy for determining cell number and viability. The technique is based on mixing cell sample with a lysis buffer to produce free nuclei. A mixture of cell suspension, lysis buffer and stabilizing buffer is then loaded into the NucleoCassette, which is internally coated with propidium iodide. The cassette is then inserted into the NucleoCounter chamber for measurement of total cell number. For nonviable cell number, the cell suspension is directly loaded into the NucleoCassette without adding the lysis buffer so that the dead cells, i.e. the cells with damaged membrane would stain by the propidium iodide.

This work aims to demonstrate the utility of the NucleoCounter for count and viability determination in mammalian cell cultures, and its suitability for routine monitoring of different cell lines. Comparison will be made with the trypan blue exclusion and flow cytometry methods.

Materials and methods

Cell lines and culture conditions

The mouse–mouse hybridoma cell line TB/C3 produces monoclonal antibody, NS0 myeloma cell line producing human IgG4 and CHO 320 producing gamma interferon were grown in batch cultures with serum supplemented and serum free media in stirred flasks at 37°C.

Trypan blue exclusion method

Classical manual counting was done using counter chamber and a binocular microscope at 10^3 magnification. Trypan blue (0.2% w/v final dye concentration) was used for viability assessment (Butler 1999). At least 12 big squares and 3 fillings of the counting chamber were assessed. For calibration experiments both sides of the haemocytometer were filled 5 times and all 18 squares were examined.

Flow cytometry method

The Stock Staining solution was prepared by mixing uniform fluorespheres (Immuno-Check, 10µm, Beckman Coulter) as internal standard with concentration of 1.1×10^5 ml^{-1} and PI with the concentration of 250 µg ml^{-1} in PBS (Phosphate Buffer Solution) at pH 7.2. For analysis on the Coulter EPICS Elite analyser, 500 µl of cell suspension was mixed with 100 µl of stock staining solution and shaken gently to get cells stained with the dye. Then immediately this sample is run through the EPICS Elite Analyser, which was equipped with an argon laser (488 nm). Red fluorescence (PI) was collected using a 635 nm band pass filter after blocking laser light with 488 nm long pass filter. Appropriate protocol was set up for the analysis for the acquired parameters: FS, log SS and PI integral. 10000 events were collected for each sample for the analysis. The evaluation of cell number was achieved by gating areas in the FS versus log SS dot plot in which beads, living cells and dead cells appear. Cell number per ml was calculated from the number of beads in relation to the total number of cell

shaped observations using the method described in Al-Rubeai et al. (1997).

Experimental design

Cell number counts and percentage of viability were obtained at 9 time points of 4 cell lines grown in batch cultures. The results are presented as average of 3 replicate counts determined by the same operator at each time point. To determine the interpersonal variation the coefficient of variation was based on the results of 3 operators analysing each sample obtained at 8 time points. Standard error is based on the results of the analysis of all cell lines at all time points by one person. Beads were used to check the accuracy of the NucleoCounter readings. Accuracy for repeated measurements was higher than 98%.

Results and Discussion

Development of a counting procedure using NucleoCounter

NucleoCounter system is composed of NucleoCassette for safe handling, NucleoCounter instrument for analysis, lysis buffer (Reagent A; used for disruption of the plasma membranes, rendering the nuclei susceptible to staining with propidium iodide) and stabilizing buffer (Reagent B; used to raise pH of the sample mixture, thereby optimizing the fluorescence of propidium iodide and stabilizing the cell nuclei). The technique is performed using portable equipment with an external chamber to measure cell number and viability without the internal movement of cell solution as the case with flow cytometry. The loading device 'NucleoCassette' is an external assembly for loading the cells into the external chamber of the NucleoCounter. It is a plastic cartridge internally pre-coated with the fluorescent dye, propidium iodide (PI), which stains the cells' nuclei. Because the NucleoCounter detects signals from the stained nuclei, and not the cells, the system operates without regard to cell size or morphology. The lysis of cells also aids the deaggregation of cell clumps.

To count cells, 150 µl of culture sample is added to 150 µl of Reagent A (lysis buffer) in a tube and is shaken properly on a vortex shaker. 150 µl of Reagent B (stabilizing buffer) is then added to that mixture and shaken thoroughly. The lysis and stabilization buffers dissolve cell aggregates and lyse cell membranes. Approximately 100µl of sample is loaded into the NucleoCassette by dipping the tip of the NucleoCassette in the sample and pushing the piston. This will produce a total cell count (based on counting stained cell nuclei) in about 30 sec. To determine cell viability, another NucleoCassette is loaded with untreated cell sample to count non-viable cells (based on counting PI stained membrane-damaged or necrotic cells). Cell viability can be determined from the two measurements: Total cells per ml and non-viable cells per ml. The viability is measured according to the following formula:

$$\%\text{Viability} = (C_t M_t - C_{nv} M_{nv})/C_t M_t * 100$$

Where, C_t = Total cell concentration in the original cell suspension (displayed result), M_t = Multiplication factor used for total cell count (as the stabilized lyzate is prepared using equal volumes of cell suspension, Lysis buffer and stabilizing buffer, the multiplication factor is 3), C_{nv} = Concentration of non-viable cells in the NucleoCassette (displayed result), M_{nv} = Multiplication factor used for the counting of non-viable cells (counting is done without dilution of the cell suspension prior to the analysis, thus the multiplication factor is 1).

Comparison of results by Trypan Blue exclusion, NucleoCounter and flow cytometry

The growth characteristics of four different cell lines grown in batch suspension culture as measured by trypan blue exclusion, NucleoCounter and flow cytometry methods are shown in Fig. 1 (a, c, e and g). Growth curves for trypan blue and NucleoCounter are looking identical, but the flow cytometry curves of hybridoma and NS0 cell numbers are different at several time points. The deviation may be due to insufficient mixing of samples before analysis or to difference in the

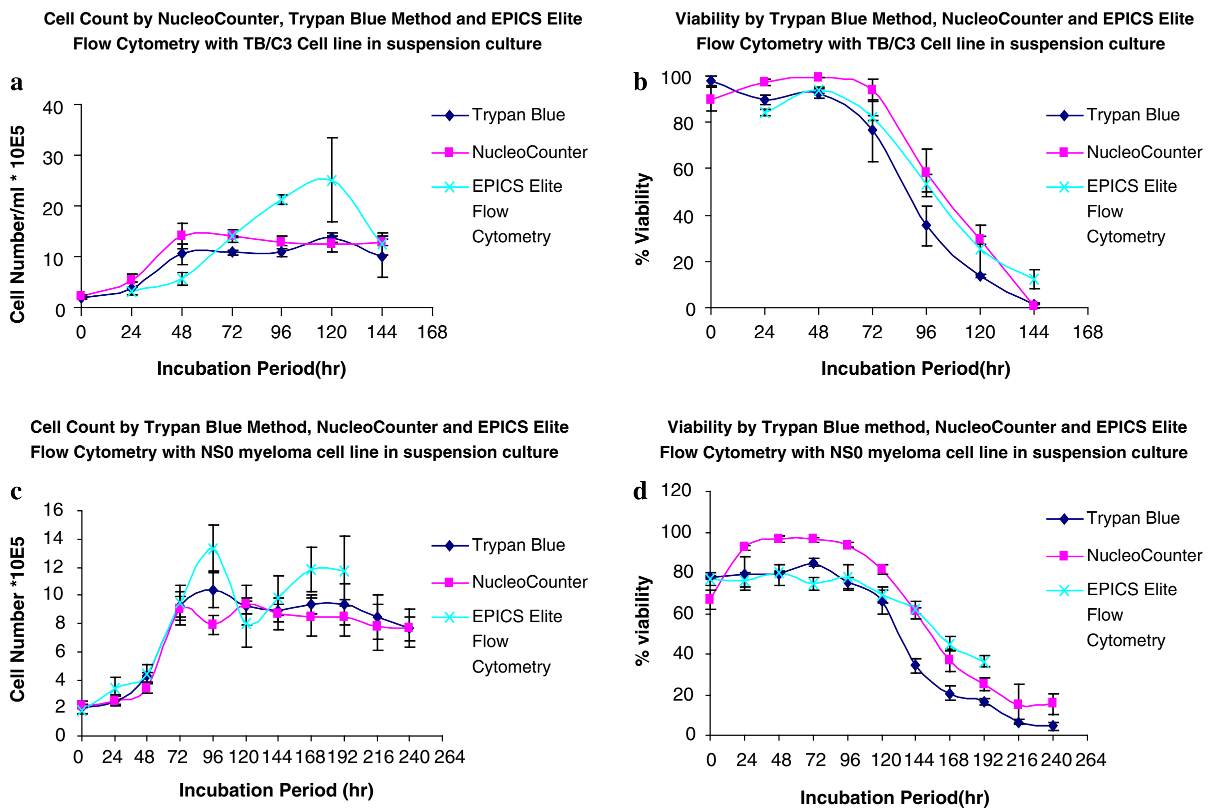

Fig. 1 Total cell number (**a**, **c**, **e** and **g**) and percentage viability (**b**, **d**, **f** and **h**) of hybridoma (**a** and **b**), NS0 myeloma (**c** and **d**) and CHO (**e** and **f**) cell lines grown in serum-supplemented batch cultures. **g** and **h** are graphic representation of the total cell number and viability, respectively of NS0 myeloma cells grown in serum free batch culture. Cell counts are made by trypan blue exclusion, NucleoCounter and flow cytomety

staining time. Exact exposure time before flow cytometry analysis is essential for accurate measurement as PI can be taken up by intact cells after prolonged exposure. Point to point curve of NucleoCounter is smoother than for both other techniques indicating a good consistency. For viability curves (Fig. 1 b, d, f and h), the results of NucleoCounter are quite consistent with the other two techniques for all the cell lines studied. In general, the results show a good agreement between the three counting methods at all phases and in high heterogeneity cultures.

Intrapersonal and interpersonal variability: NucleoCounter and trypan blue method

Figure 2 shows the mean coefficient of variations (CVs) as indicators of the variation resulted from repeated analysis of cell samples by the manual trypan blue method in comparison to the automated NucleoCounter method, when one person is taking triplicate reading from the same sample. The average coefficient of variation of all readings is higher with trypan blue, specifically in the exponential and stationary phase, where heterogeneity is likely to increase indicating a higher precision in the NucleoCounter method and the negligible influence of the operator on counting and viability measurements.

Counting cells with a haemocytometer is regarded as being subject to inter-observer variation. Reasons for such variations may include; counting few squares and too few cells; uneven distribution of cells in the sample (sampling error); incorrect dilution; overfilling or variation of haemocytometer filling rate (Al-Rubeai et al. 1997). The apparent discrepancy between the two methods may also be due to the use of different stains. Stains exhibit different properties; a dying cell membrane may be permeable to one stain but not to another.

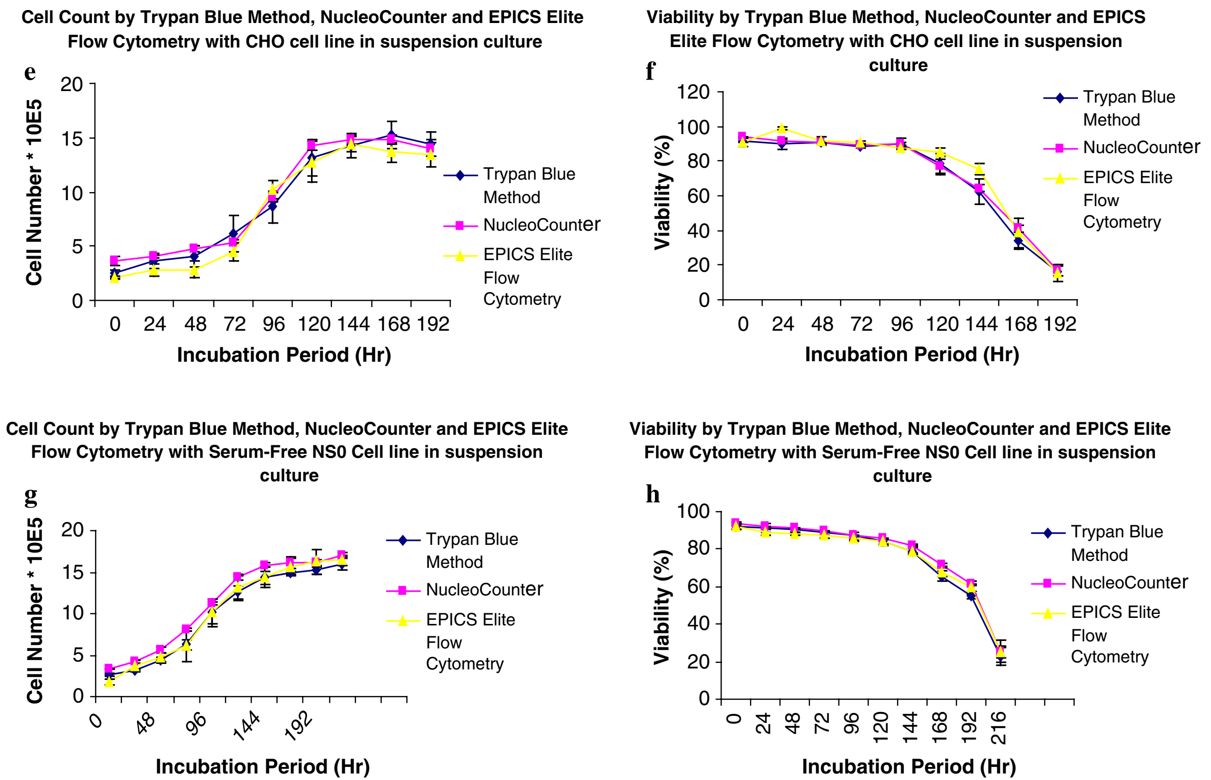

Fig. 1 continued

Figure 3 shows the mean standard error which represents the data set of standard deviations of all triplicate readings taken out throughout the incubation period of hybridoma cultures. The mean standard error for both total cell number and viability using the NucleoCounter was significantly lower than that obtained by the other two methods indicating lower operator dependency and better reproducibility and objectivity.

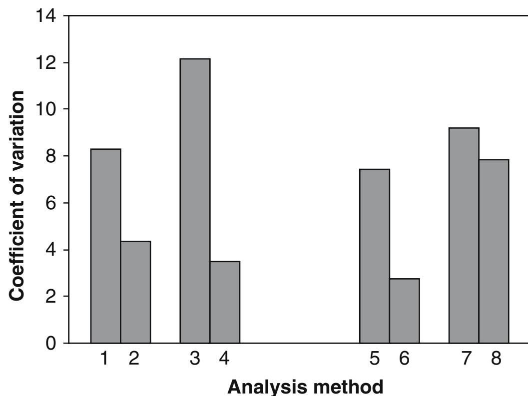

Fig. 2 Mean coefficient of variations (CVs) of repeated measurements obtained by trypan blue (1,3,5,7) and NucleoCounter (2,4,6,8) methods for cell density (1,2,3,4) and viability (5,6,7,8) of hybridoma cells. 1,2,5 and 6: Intrapersonal variation; 3,4,7 and 8: Interpersonal variation

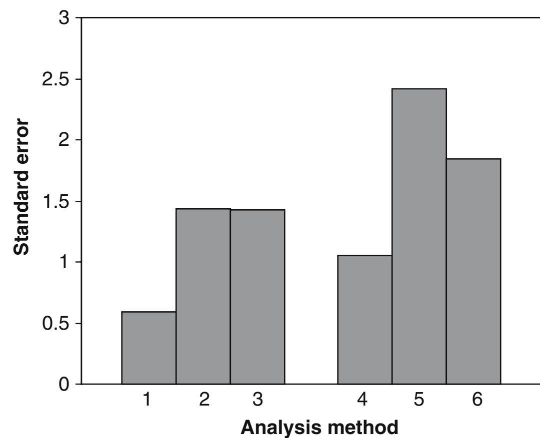

Fig. 3 Mean Standard error of repeated measurements obtained by NucleoCounter (1,4), trypan blue (2,5) and flow cytometry methods (3,6) for cell density (1,2,3) and viability (4,5,6) of hybridoma cells

Considering the speed of analysis, we found that manual assessment of cell number and viability for one sample may take approximately 15 min. This depends on the number of counted cells, squares and the experience of the operator (Al-Rubeai et al. 1997). In comparison, the flow cytometric and NucleoCounter measurements were only two pipetting steps followed by the analysis, both analysis takes about one minute.

Conclusion

It is demonstrated that the NucleoCounter is a rapid, precise and objective tool for routine measurement of cell density and viability in animal cell cultures. It yields comparable results to standard methods with a higher degree of reproducibility between experiments and operators which makes it an excellent method for on line monitoring of cell culture processes.

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