A Simple Method for Counting Small Numbers of Cells by Flow Cytometry

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We describe a flow cytometric method for the quantitation of small numbers of cells that facilitates proliferation studies in microcultures. A constant number of fluorescent latex microspheres/sample is added to single cell suspensions prepared from the cultures. By flow cytometric analysis, cells are easily distinguishable from the microspheres and can be quantitated on the basis of their light scattering and fluorescence properties in contour plots. As the number of microspheres/sample is known, the relative proportions of cells and microspheres, respectively, can be converted into absolute cell numbers. This quick method is useful for any type of studies where cell numbers $< 1 \times 10^5$ in a small volume are to be determined.

Introduction

Precise enumeration of small numbers of cells in monolayer or suspension cultures is often required in cell biological studies. In our current analyses, the proliferative characteristics of fetal rat brain cells (FBC) had to be determined in microcultures of $\leq 1 \times 10^5$ cells. Existing procedures for counting small numbers of cells can be subdivided into two general groups. The first group includes fixation or homogenization of the cells with subsequent staining of DNA [1–3] or protein [4]. These methods lack precision since in the case of DNA staining no distinction can be made between cells in different phases of the cell cycle, and protein contents vary between both cell types and cell cycle phases (*e.g.*, the heterogeneous cell populations of the brain). The second group of procedures include the counting of cells by hemocytometer (precise only for cell concentrations $>10^5$/ml) or automatic counting by Coulter Counter [5]. In the latter case, small cell numbers may be determined but no distinction can be made between dead and viable cells. We, therefore, developed a quantitation method for small numbers of viable cells ($\geq 10^3$ cells/sample) using a fluorescence-activated cell sorter (FACS) and fluorescent microspheres (beads) as an internal counting standard. This method has the following advantages: (i) very small numbers of heterogeneous types of viable primary cells can be counted; (ii) neither fixation nor staining of cells is required; and (iii) cell counts are obtained quickly.

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Materials and Methods

Primary cultures of FBC (prenatal day 18) were prepared as previously described [6, 7] and grown as monolayers in 48-well Costar cell culture plates (Tecnomara, Fernwald, F.R.G.) containing 200 µl/well (0.78 cm^2) of modified Eagle-Dulbecco medium [8] supplemented with 10% fetal calf serum (FCS, Dunn Labortechnik, Asbach, F.R.G.; Lot 01511/87), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For flow cytometric analysis, cells were removed from the wells by incubation with 200 µl/well of 0.2% (w/v) trypsin in phosphate-buffered saline (PBS) containing 0.02% (w/v) EDTA at 37 °C for 5 min. After microscopic control, cells were collected in siliconized Eppendorf tubes (Sarstedt, Nümbrecht, F.R.G.). Trypsin digestion was interrupted by addition of 100 µl FCS/tube and the samples were filled up to 1 ml with PBS and kept on ice until analysis. Fluorescent latex microspheres (Immuno-Check, Epics Division of Coulter Corp., Hialeah, Fla., U.S.A.; Lot No. 5248; diameter, 10 µm; stock solution 1×10^6 microspheres/ml) were counted in a hemocytometer and thereafter diluted in PBS. Immediately prior to flow cytometric analysis, a constant number of $1-5 \times 10^4$ FITC-labeled microspheres were added per sample.

For determination of the limiting cell concentration, a stock suspension of freshly dissociated FBC was counted microscopically in a hemocytometer and analyzed by flow cytometry at different dilutions after addition of 2×10^4 microspheres/sample. The lowest cell number analyzed was 1×10^3/sample. A mixture of 3 different types of latex microspheres (A: fluorescein beads Immuno-Check; B: fluorescein beads; Flow Cytometry Standards Corp.; Lot No. 040189; diameter, 9.5 µm; C: fluorescein beads FULLBRIGHT; Epics Division of Coulter Corp.; Lot No. 4130; di-

ameter, 5.33 μm) were tested for adhesion to the plastic tubes and syringes and for sedimentation during the time of analysis (8 min; sample histograms were recorded and stored at 30 sec intervals).

Flow cytometric analysis was performed using a modified FACS II instrument (Becton Dickinson, Palo Alto, Cal., U.S.A.). Excitation of fluorescein (FITC) and phycoerythrin (PE) dyes as well as the light for scattering were provided by an argon laser Innova 90-6 (Coherent, Palo Alto, Cal., U.S.A.) at 488 nm. Three parameters were measured simultaneously, *i.e.*, red (PE) and green (FITC) fluorescence and forward scatter. Red and green fluorescence were measured with bandpass filters at 585 nm and 530 nm, respectively.

Results and Discussion

The fluorescent microspheres used as an internal counting standard required two main characteristics: (i) a fluorescence intensity sufficient to distinguish them from the cells; and (ii) a statistical distribution in the samples (*i.e.*, a sedimentation rate comparable with the cells) during the time of analysis. The last point guarantees the homogeneous analysis of the suspension. The test experiments revealed that two of the 3 types of microspheres chosen were suited for the present method. As shown in Fig. 1, all types of microspheres could be measured relatively stably over a period of 8 min. But type B microspheres proved to be unsuitable, since their fluorescence intensity in measurements with admixed FBC was too weak to be distinguishable from the autofluorescence of the cells (not shown). This distinction is a fundamental requirement for the evaluation procedure (see below). In contrast, both the type A and type C microspheres displayed a high fluorescence intensity and could easily be separated from the FBC. As type C microspheres are no longer commercially available, type A microspheres were used for all further analyses. Fig. 2 shows the evaluation of the data recorded for a mixture of FBC and type A microspheres. On the basis of light scatter (size) and fluorescence intensity, single type A microspheres and viable single cells were marked by two masks in the contour plot (Fig. 2C). The relative proportions of cells obtained from the single cell peak defined by the Gaussian function of the scat-

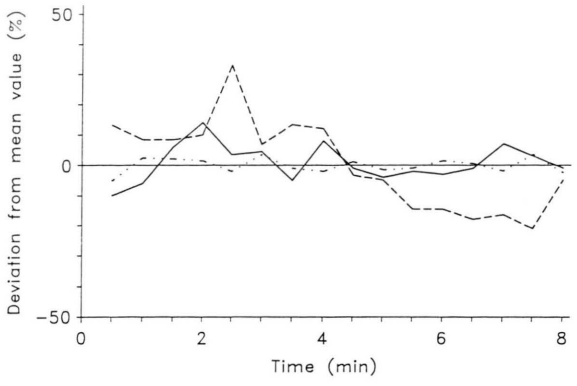

Fig. 1. Sedimentation analysis of a mixture of 3 types of latex microspheres over a period of 8 min. The stability of the measurements is demonstrated by the deviation of the data (each value is a mean of 3 independent measurements) from the mean value for all samples (0% line). Sample histograms were recorded and stored at 30 sec intervals. —— Fluorescein beads (Immuno-Check; type A microspheres); – – – – fluorescein beads (type B microspheres); –··–··–·· fluorescein beads (FULLBRIGHT; type C microspheres).

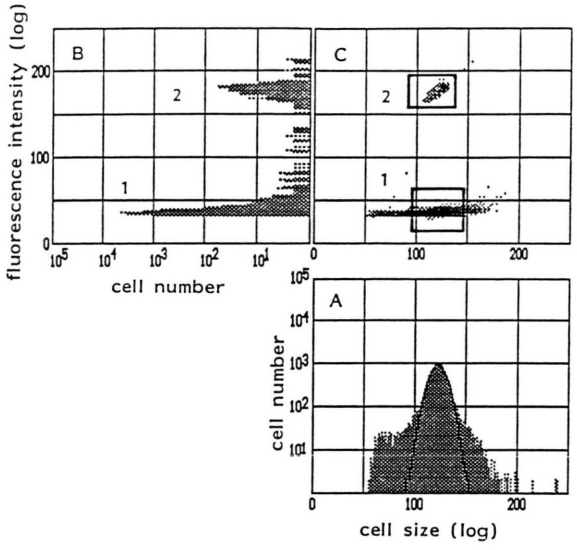

Fig. 2. Evaluation of computer histograms: (A) Scatter distribution of fetal rat brain cells; the single cell peak is marked by a Gaussian function (dark line); (B) fluorescence distribution of cells (1) and microspheres (2); (C) contour plot with defined masks for single cells (1) and microspheres (2), respectively. Particle counts in the two masks yield the fractions C_{FACS} and S_{FACS}, respectively, used in the evaluation formula.

ter distribution (Fig. 2A) can be correlated with the sum of the relative proportions in the masks of single cells and microspheres in the contour plot. Both cell debris and cell aggregates were thus gated out on the basis of their light scattering characteristics. The relative proportions of particles in both masks were determined by computer. In addition, double-fluorescence analysis with the DNA-specific dye propidium iodide (PI; red fluorescence) was performed in order to obtain a second criterion permitting the elimination of signals from cell debris still present in the scatter distribution of the viable cells. PI selectively enters dead or damaged cells so that these cells become readily distinguishable from viable autofluorescent cells and green microspheres, respectively. Samples analyzed after incubation with PI showed negligible PI-positive debris in the size range of single cells, and could, therefore, be evaluated on the basis of the scatter signals. By this extension of the method, only one excitation line is needed for flow cytometric analysis. In this case the position of the fluorescent microspheres in the contour plot (Fig. 2C) was determined without cells in the sample.

The present procedure resembles, in principle, a method described by Stewart and Steinkamp [9]. However, the latter method includes fixation of the cells prior to staining with the DNA-specific dye Hoechst 33342 and green or red fluorescent microspheres as the internal counting standard. A dual laser system is thus required, since a single UV excitation line would cause interference of the Hoechst 33342 fluorescence with green spheres in the green fluorescence channel. Moreover, red fluorescent spheres cannot be excited in combination with Hoechst 33342 using a single laser line.

In the present procedure, the total number of cells per sample is calculated by the formula:

$$C_n = \frac{C_{FACS}}{S_{FACS}} \times S_n,$$

where C_n is the total number of cells per sample, C_{FACS} is the fraction of single cells counted by flow cytometry, S_{FACS} is the fraction of single microspheres counted by flow cytometry, and S_n is the total number of fluorescent microspheres added to the sample. At variance with the procedure of Stewart and Steinkamp [8] the sample volume can be neglected since total cell numbers per sample but not cell concentrations are determined. Cell numbers $\geq 1 \times 10^3$ could be analyzed with a mean standard deviation of $\pm$ 5% (see Fig. 3).

Fig. 3 shows the titration of the total number of FBC that could be counted precisely after addition of a constant number (2×10^4) of FITC-labeled type A microspheres per sample. Absolute cell numbers per dilution were calculated on the basis of calibration with FBC from a stock solution counted by hemocytometer. Even cell numbers $< 10^3$/sample may be counted precisely, if the number of added microspheres per sample is $< 2 \times 10^4$ (the absolute number of cells to be counted should be $\geq$ 5% of the absolute number of microspheres added).

The present procedure (contrary to that described in ref. [9]) is only of limited value when cell suspensions are to be analyzed that contain a significant amount of cell aggregates. While cell aggregates stained with Hoechst 33342 are easily detectable by DNA analysis, discrimination is not possible between aggregates of two cells with a G_1/G_0 DNA content and single cells in the G_2 + M phase of the cell cycle. In the present method, single cells were counted up to a distinct channel in the scatter distribution (Fig. 2A). In cell suspensions containing cell aggregates, cells may be separated by including 2 U/ml of DNAse I and 6 mM $MgCl_2$ in the PBS added to the cell suspension aft-

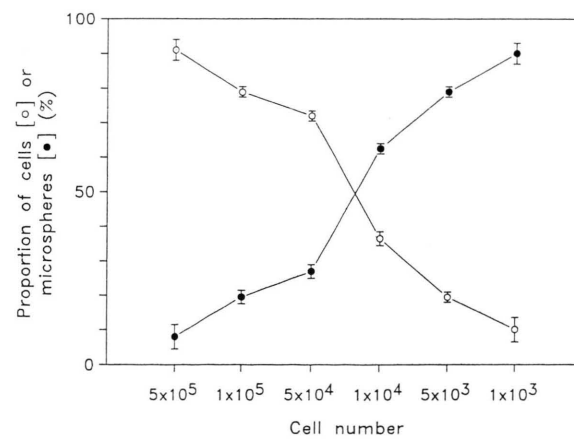

Fig. 3. Titration of the number of cells counted. Nearly 10% of the recorded events were due to cell debris gated out as shown in Fig. 2. Each value is a mean of three independent measurements (bars indicate standard deviation). ○—○ Cells; ●—● microspheres.

er trypsinization. DNAse I digests the DNA of dead or damaged cells that causes clotting of cells in suspension. By this treatment most of the cell aggregates can be disintegrated and cells usually appear in a single cell peak.

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