Benchmarks

Counting nuclei released from microcarrier-based cultures using pro-fluorescent nucleic acid stains and volumetric flow cytometry

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Counting nuclei released from intact cells is a convenient and reliable approach to assess cell density during microcarrier-based culture of adherent cells. However, commonly used methods for counting nuclei, such as crystal violet staining and quantification with a hemocytometer/automated imaging system or a Coulter counter, are imprecise, laborious and, limited in throughput. Here, we describe the use of high-affinity pro-fluorescent nucleic acid stains and volumetric flow cytometry for automated counting of nuclei released from cells attached to microcarriers with improved precision and high sample throughput. This simple procedure facilitates rapid and precise assessment of cell attachment, survival, and proliferation on microcarriers, and can provide information about the cell cycle, all without the need for cell detachment. Consequently, various microcarrier-based applications, from small-scale multi-factor experiments to large-scale functional genetic screens and clinical/industrial cultures, could be enhanced by this approach.

Counting nuclei released from cultured cells has proven to be a reliable method for cell enumeration since first described in 1951 by Sanford et al. (1). The method was subsequently

adapted to monitor cell attachment and proliferation in microcarrier-based cultures of adherent cells, avoiding time-consuming detachment (e.g., by trypsinization) (2). Techniques for the

release of nuclei from cells attached to various types of microcarriers involve the use of a solution of hypotonic citric acid containing crystal violet, for effecting cell lysis and staining nuclei, respectively, with or without 0.1-1% (v/v) Triton X-100/IGEPAL CA-630 detergent for reducing the size of cell debris (2-5). Nuclei in the suspension are then often counted using image-based methods (e.g., hemocytometer or automated cell counter) or methods based on aperture electrical resistance measurement (e.g., Coulter counter) (4-7). However, these counting methods are suboptimal. For example, image-based counting is laborious, time-consuming, and imprecise due to overestimation of nuclei numbers resulting from difficulty in distinguishing them from debris and to the typically small number of nuclei quantified per sample. Although a Coulter counter can process more nuclei per sample than image-based counting, it distinguishes nuclei from debris based on particle size, which may also cause overestimation of nuclei numbers as some debris or debris aggregates are similar in size to nuclei. Lastly, neither procedure allows truly automated counting compatible with high-throughput sample processing (e.g., reading in a 96-well plate format).

Here, we present a modified method for counting nuclei that incorporates a high-affinity pro-fluorescent nucleic acid stain and volumetric flow cytometry to achieve automated sample acquisition with improved precision. Nuclei released from microcarrier-based cultures of a human dermal microvascular blood endothelial cell (MVBEC) line (catalog #XSEB113C1; Lonza, Mount Waverley, Victoria, Australia) were transferred into 5 mL polystyrene tubes or 96-well plates for staining and acquisition using a flow cytometer with volumetric sensor (see protocol in the Supplementary Material). Initially, we evaluated SYTOX Red (Life Technologies, Mulgrave, Victoria, Australia) to stain nuclei by virtue of its high molar absorptivity, low intrinsic fluorescence, high affinity for DNA, and large fluores-

METHOD SUMMARY

Here, we present a procedure for precise counting of nuclei released from cells attached to microcarriers using a high-affinity pro-fluorescent nucleic acid stain and volumetric flow cytometry.

Figure 1. Analysis of nuclei released from microcarrier-based cultures of human dermal microvascular blood endothelial cell (MVBECs) using SYTOX Red and volumetric flow cytometry. (A,B) Gating strategy for volumetric flow cytometry–based counting of nuclei using SYTOX Red. Debris was first excluded using forward scatter (area) versus side scatter (area) plots (data not shown). A control sample of unstained nuclei (A) was used to establish the lower threshold for SYTOX Red positivity in (B). The contour plots display a 5% interval. Total events acquired = 20,000. Flow rate was high. (C,D) Gating strategy for flow cytometry–based analysis of the cell cycle using propidium iodide–stained nuclei. After first excluding debris (as above; data not shown), aggregates and debris were gated out (C), and the population of single nuclei was visualized on a propidium iodide intensity histogram (D). Cell-cycle analysis was performed using FlowJo software (version: 10.0.8r1; FlowJo, LLC, Ashland, OR). Total events acquired = 10,000. Flow rate was medium. Contour intervals in (C) are 5%. Nuclei populations represent means from three replicate counts of the one sample $\pm$ SEM.

cence enhancement upon binding. When excited at 633 nm, we observed a population of nuclei with far-red fluorescence that allowed a simple gating strategy involving forward scatter, side scatter, and SYTOX Red fluorescence intensity to resolve nuclei from debris in the crude cell lysate (Figure 1A and 1B). With this information, plus the corresponding sample volume, nuclei concentration could be calculated (see protocol in the Supplementary Material). In addition, analysis of the cellular DNA content in the same sample was performed using RNase treatment and propidium iodide (PI) (Life Technologies). When debris and aggregates were excluded (Figure 1C), a histogram of the PI-stained nuclei resolved those with a DNA content of $2N$ (Figure 1D, first peak, G_0 and G_1 phases) and $4N$ (Figure 1D, second peak, G_2 and M phases). Nuclei with an intermediate DNA content represent those in S phase (Figure 1D). Quantitative analysis of the cell cycle was performed using the Watson Pragmatic algorithm (8) (Figure 1D).

Next, we evaluated whether our method is compatible with different nucleic acid stains. Counting results using the other four stains from the SYTOX product family (SYTOX

Figure 2. Method evaluation. (A) Quantification of human dermal microvascular blood endothelial cell (MVBEC) nuclei concentration using SYTOX Blue, SYTOX Green, SYTOX Orange, SYTOX AADvanced, propidium iodide, or SYTOX Red, and volumetric flow cytometry. Nuclei concentrations represent means from three replicate counts of one sample. Error bars represent mean $\pm$ SEM (SYTOX Blue: 176,798 $\pm$ 1298; SYTOX Green: 176,472 $\pm$ 1005; SYTOX Orange: 172,855 $\pm$ 2168; SYTOX AADvanced: 176,811 $\pm$ 444; PI: 178,474 $\pm$ 807; SYTOX Red, 176,078 $\pm$ 2814). (B,C) Gating strategy for volumetric flow cytometry–based counting of nuclei released from HEK 293T cells on microcarriers using SYTOX Red. The contour plots display a 5% interval. Total events acquired = 20,000. Flow rate was high. (D) Observed and expected nuclei concentrations from MVBECs as determined by volumetric flow cytometry using SYTOX Red. A dilution series was prepared, and the concentration of nuclei was determined. Each observed concentration was multiplied by its corresponding dilution factor to obtain an estimate of the concentration in the undiluted sample. The average of these estimates was considered the concentration of the undiluted sample. Expected concentrations were calculated by dividing the concentration in the undiluted sample by the dilution factor. Each sample was counted twice, and the average was plotted. The dashed line indicates the line of identity (i.e., perfect agreement between the observed and estimated counts).

Blue, SYTOX Green, SYTOX Orange, and SYTOX AADvanced; Life Technologies) and PI showed that the concentration of nuclei in a test sample measured using all five stains was very similar to that determined using SYTOX Red (coefficient of variation across the six stains, 1.68%) (Figure 2A). In addition, analysis of the cellular DNA content using SYTOX stains gave similar results to PI (data not shown). These findings indicate good compatibility of these stains with our method. Finally, we successfully counted nuclei released from human embryonic kidney 293T (HEK 293T) cells on microcarriers using SYTOX Red (Figure 2. B and C), demonstrating that our method is not restricted to MVBECs on microcarriers.

To determine the dynamic range of our method, a wide range of dilutions of nuclei released from MVBECs on microcarriers was prepared, and concentrations were measured using SYTOX Red and volumetric flow cytometry. We plotted the observed concentrations against the expected concen-

trations to determine where the data started to deviate from linearity (Figure 2D). In close agreement with a previous observation regarding the appropriate dynamic range for counting 7-amino-actinomycin D (7-AAD)-stained nuclei by flow cytometry (6), our data started to deviate from the line of identity at approximately 5×10^5 nuclei/mL. Therefore, we recommend that nuclei to be counted using our method be diluted below this value.

To the best of our knowledge, the combination of flow cytometry and pro-fluorescent nucleic acid stains presented here has not previously been used to count nuclei released from cells on microcarriers. Our modifications to the common methods of counting nuclei released from cells on microcarriers are significant in that they are applicable to multiple cell types, allow automation and high sample throughput with consequent labor and time savings, can provide information about cell cycle distribution using the same sample, and deliver much improved precision. The latter is a direct consequence of the ability of our method to process many more nuclei per sample than visual counting and the fact that count data display characteristics of a Poisson distribution (9). Pisanía et al. (6) examined three methods for the enumeration of nuclei released from insulin-secreting cell lines and pancreatic islets, including volumetric flow cytometry after staining with 7-AAD. However, in that study, only 1000 nuclei per sample were processed (versus ≥ 5000 nuclei per sample processed using our method), 7-AAD was the only dye tested, and the suitability of the method for counting nuclei released from cells on microcarriers was not tested (6). Hence, a variety of microcarrier-based tasks, such as performing parallel small-scale cultures to evaluate the roles of multiple factors in culture performance (5), monitoring large-scale pooled functional genetic screens (10,11), developing culture systems for stem cells to enable regenerative therapy (7,12), and clinical/industrial production of viruses, vaccines, and antibodies (3,13) may benefit from the application of our method.

Author contributions

M.Y.C.H. and M.M.H. conceived the study. M.Y.C.H., M.M.H., and R.R. designed experiments. M.Y.C.H. performed experiments. All authors analyzed data. M.Y.C.H. prepared the manuscript with assistance from M.M.H., S.A.S., and R.R.

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Competing interests

The authors declare no competing interests.

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