Transient gene expression in CHO cells monitored with automated flow cytometry

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Abstract Transient gene expression is frequently used in industry to rapidly generate usable quantities of a protein from cultured cells. In gene therapy applications it is used to express a therapeutic protein in vivo. A quantitative assessment of the expression kinetics is important because it enables optimization and control of culture conditions for higher productivity. Previous experimental studies show a characteristic peak in average protein expression per cell after transfection followed by an exponential decrease of the expressed protein. Here, we show that the exponential decrease in single cell expression of enhanced Green Fluorescent Protein (eGfp) occurs in discrete steps. We attribute this to the absence of plasmid replication and to symmetric partitioning of plasmid and eGfp between dividing cells. This is reflected in the total eGfp in the bioreactor, which increased at a constant rate throughout the experiment. Additionally, the

data provide a detailed time course of cell physiology during recovery from electroporation. The time course of cell physiology precisely indicates when the culture shifts growth phases. Furthermore, the data indicate two unique stationary phases. One type of stationary phase occurs when proliferation ceases while cells decrease their cell size, maintain granularity, and mean eGfp content decreases. The second type occurs when proliferation ceases while cells increase their cell size, increase granularity, and surprisingly maintain eGfp content. The collected data demonstrate the utility of automated flow cytometry for unique bioreactor monitoring and control capabilities in accordance with the US Food and Drug Administration's Process Analytical Technology initiative.

Keywords Transient gene expression · Automated flow cytometry · Process analytical technology · Chinese hamster ovary · Bioreactor monitoring · Single cell heterogeneity

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Nomenclature

CHO Chinese hamster ovary
FSC Forward light scatter
SSC Side light scatter
CV Coefficient of variation
eGfp Enhanced green fluorescent protein
N Non-fluorescent cell number
F Fluorescent cell number

μ	Specific proliferation rate (h^{-1})
μ_F	Specific proliferation rate fluorescent fraction
μ_N	Specific proliferation rate non-fluorescent fraction
μ_F^*	Effective proliferation rate fluorescent fraction
μ_N^*	Effective proliferation rate non-fluorescent fraction
$k_{d,F}$	Death rate fluorescent fraction
$k_{d,N}$	Death rate non-fluorescent fraction
k_T	Non-fluorescent to fluorescent transition rate

Introduction

A significant problem in mammalian cell culture is the direct assessment of the concentration and composition of the biomass. It is well recognized that considerable single cell variability exists in a cell culture and that this heterogeneity affects culture performance. Such culture variability is also likely to a large extent responsible for variations that are observed in the final product obtained. Therefore, new and improved measuring methods, able to detect the single cell variability, could considerably enhance the monitoring and control of manufacturing processes ultimately resulting in better quality products. The Food and Drug Administration has recently recognized the practical value of this approach by establishing the Process Analytical Technology (PAT) initiative aiming at more reliable products on the basis of better control and understanding of biological–pharmaceutical manufacturing processes.

The monitoring and control of a cell cultivation process is generally limited by the data that can be collected. Typically, measured online culture process variables only characterize bulk properties (i.e. OD, OUR, DO_2 , pH, etc) while offline measurements such as cell counts, viability, etc are assessed relatively infrequently. Furthermore, most of these measurements cannot determine the inherently heterogeneous nature of a culture. Culture heterogeneity is caused by factors such as the cell cycle, incomplete mixing, genetic varia-

tion, and stochastic events. Therefore, it is of considerable interest to detect this heterogeneity, to monitor it online, and ultimately to control it with appropriate process conditions. To accomplish this, we developed an automated flow cytometry system (Abu-Absi et al. 2003) that has already been shown to be useful for a variety of applications including monitoring of microbial (Kacmar et al. 2004) and mammalian cell cultures (Kacmar and Srienc 2005). Automated flow cytometry can provide online cell number and viability, with the ability to discriminate sub-populations even if they are present as a small fraction of the total cell population. It represents a general methodology that can provide information on virtually any characteristics of the sub-populations for which a flow cytometry stain exists.

Cell heterogeneity is expected to significantly affect the transient gene expression characteristics of a culture. However, it has been little studied likely due to the limited methods that are available. Reported transient gene expression studies focus on either the population averaged properties of the culture over time, or on the optimization of bulk culture variables (Derouazi et al. 2004). Few studies address the issue of culture heterogeneity during transient gene expression (Keith et al. 2000). In addition to the inherent sources of heterogeneity in a culture undergoing transient gene expression, there is also heterogeneity induced by the specific transfection procedure. There are several protocols used to transfect mammalian cells; of these electroporation is a common non-viral technique (Cegovnik and Novakovic 2004). The efficiency and the effects of electroporation are expected to greatly depend on the initial physiological state of the cells. Here, we use automated flow cytometry to compare and to contrast the physiological response after electroporation of a culture in stationary phase to a culture in exponential phase. The results offer a detailed insight into the population dynamics during transient gene expression. Furthermore, it is a further demonstration of automated flow cytometry as a tool to obtain detailed population data that directly comply with the goals of the PAT initiative.

Materials and methods

Cell line and growth medium

Serum free CHO cells of the strain CHO-S (Invitrogen, Carlsbad, CA) were used. Cells from a working cell bank, stored in liquid nitrogen, were thawed at 37°C and placed in a T-flask (75 cm², Corning, Inc., Corning, NY) containing 25 ml of CHO-S-SFM II medium (Invitrogen) at a concentration of 1×10^5 cells ml⁻¹. The cells were incubated at 37°C in air containing 7.5% carbon dioxide. After being passed once into 50 ml of medium in the same size T-flask, the cells were passed a third time into a total volume of 300 ml in a 500 ml spinner flask (Corning, Inc., Corning, NY).

Transfection procedure

Transfections were performed by dividing an inoculum into aliquots containing 2×10^7 cells and then centrifuging each aliquot. The resulting cell pellets were resuspended in CHO-S SFM II medium with 100 µg plasmid peGfp-N1 (Clontech) for a final electroporation volume of 800 µl. Plasmid peGfp-N1 was extracted from *E. coli* using Qiagen's Maxiprep column. Each 800 µl aliquot was transferred to an electroporation cuvette with a gap of 0.4 mm (Fisher, Pittsburgh, PA) and incubated on ice for 10 min. The aliquots were then electroporated with an exponentially decaying pulse at a voltage of 330 V, and an average time constant of 18 ± 0.5 ms. The electroporated aliquots were then incubated on ice for 10 min, recombined into 20 ml of CHO-S medium, and used to inoculate bioreactors.

Bioreactor operation

A 2-l bioreactor (LH Fermentation) with a working volume of 1-l was operated at 37°C. The bioreactor was agitated at a rate of 100 rpm using a six blade disk impeller with a 5 cm diameter. Air and CO₂ were sparged to control DO and pH, respectively. The DO remained above 80% during the duration of the experiment and the pH remained at 7.2 ± 0.05 . One hour after inoculation, sodium penicillin G (Sigma, St. Louis, MO)

and streptomycin sulfate (Sigma, St. Louis, MO) were added to a final concentration of 0.17 and 68.6 mM, respectively. On day 3, 400 ml of fresh CHO-S SFM II medium supplemented with penicillin and streptomycin were batched into Culture E.

Sampling and analysis

Samples were automatically withdrawn from the bioreactor every 21 min and processed by the previously described automated cell preparation system (Abu-Absi et al. 2003; Zhao et al. 1999). A commercial version of such sample preparation system has been recently developed by MSP Corp, Shoreview, MN (<http://www.msppcorp.com>). For each sampling cycle, the automated cell preparation system loaded a 1.5 mL of sample directly from the bioreactor into a 46 µL sample loop. The 1.5 mL of sample was sufficiently large to completely purge and replace the contents of the sample loop. The content of the sample loop was then injected into the flow cytometer for analysis at a flow rate of 13 µL min⁻¹. Next, a second 1.5 mL cell sample was loaded into the stirred microchamber and subsequently stained with propidium iodide for determination of cell viability. The stained cells were then washed with phosphate buffered saline. The stained cells in the microchamber were then loaded into the sample loop and the content of the sample loop was injected into the cytometer for analysis. No additional sample preparation steps are required to eliminate cell aggregates, since the fraction of cell aggregates (defined as three or more cells touching each other) as determined by microscopic examination was less than 2% of the original sample (data not shown).

Flow cytometry

A FACS Calibur (Becton-Dickinson Immunocytometry System, San Jose, CA) flow cytometer was used for the analysis of cells. A 15 mW laser (Spectra-Physics, Mountain View, CA) with a wavelength of 488 nm was used for excitation. Data from the forward scatter diode and side scatter photomultiplier tube were digitized with a linear scale. Data from the photomultiplier tube

collecting the green fluorescence (530 nm) of the cells was digitized on a logarithmic scale. Throughout the experiment, the cytometer was set on a sample flow rate setting of 'High'. This setting sets the air pressure applied to the contents of the sample loop in the automated sampling system, which in turn specifies the sample flow rate into the cytometer. The 'High' sample flow rate setting resulted in an average sample flow rate of $13 \mu\text{L min}^{-1}$ and an event rate of up to 580 cells s^{-1} .

Cell proliferation model

Apoptosis, proliferation, and the transition of non-fluorescent to fluorescent cells due to plasmid expression can preferentially affect the fluorescent and non-fluorescent sub-populations of the viable cell population. Equations 1, 2 describe these dynamics:

$$\frac{dF}{dt} = (\mu_F - k_{d,F})F + k_T N = \mu_F^* F + k_T N \quad (1)$$

$$\frac{dN}{dt} = (\mu_N - k_{d,N})N - k_T F = \mu_N^* N - k_T F \quad (2)$$

where F is the fluorescent cell number, N is the non-fluorescent cell number, μ_F and μ_N are the proliferation rates of the fluorescent and non-fluorescent sub-populations, respectively, $k_{d,F}$ and $k_{d,N}$ are the cell death rates of the fluorescent and non-fluorescent sub-populations, respectively, and k_T is the transition rate between the fluorescent and non-fluorescent cells. The terms $(\mu_F - k_{d,F})$ and $(\mu_N - k_{d,N})$ can be combined to yield an effective growth rate μ_F^* and μ_N^* , respectively.

eGfp production model

The average single cell eGfp content is obtained by taking the first moment of the distribution of eGfp content of transfected cells:

$$\bar{x} = \int_{TT}^{\infty} x f(x) dx \quad (3)$$

where $\bar{x}$ is the average single cell eGfp content, the transfection threshold (TT) is the level of

green fluorescence slightly above the auto-fluorescence of non-transfected CHO cells, x is the eGfp content, and $f(x)$ is the distribution of eGfp content. Since eGfp is an intracellular protein, multiplication of the fluorescent cell number in the bioreactor with the average single cell fluorescence results in the total eGfp content in the culture at each time point:

$$X(t) = \bar{x}(t)N(t) = \bar{x}(t)N_0 \exp(\mu t) \quad (4)$$

where $X(t)$ is the total eGfp content, $N(t)$ is the fluorescent cell number, N_0 is the initial transfected cell number, and μ is the growth rate of the culture.

As observed in this study, the total eGfp content increases linearly (Fig. 5). It is assumed that the rate of increase of the total eGfp content is proportional to the initial plasmid copy number in the initially transfected population. Therefore, the rate of eGfp production is subsequently proportional to the initial number of transfected cells:

$$X(t) = k_1 P_0 t = k N_0 t \quad (5)$$

where k and k_1 are proportionality constants, P_0 is the initial plasmid copy number, and N_0 the initially transfected cell number.

Substituting Eq. 5 into Eq. 4, solving for $\bar{x}(t)$ and taking the natural log of both sides yields:

$$\ln(\bar{x}(t)) = \ln(k) + \ln(t) - \mu t \quad (6)$$

which describes the time course of the mean single cell eGfp content during the expression experiment.

Results

Proliferation characteristics

The detailed single cell data from frequent sampling reveal significant differences depending on culture history and culture conditions. The transient gene expression characteristics of a culture started from an exponential inoculum (culture E) was compared to a culture started

Fig. 1 Apoptotic population. FSC vs SSC comparison for a sample point during bioreactor operation. Apoptotic cells are defined as having decreased FSC and increased SSC as previously described (Schwartz and Osborne 1995)

from an inoculum in stationary growth phase for at least 12 h (culture S). Each inoculum was divided into six 45 ml aliquots and centrifuged. The resulting cell pellets were independently re-suspended in medium containing plasmid, and at time zero, each of the aliquots was electroporated as described in the methods section. The fraction of viable cells of the cultures immediately before electroporation was 95 and 92% for cultures E and S, respectively. The electroporated aliquots were then recombined, and used to inoculate a bioreactor that was monitored by automated flow cytometry.

With automated flow cytometry, the time course of apoptotic cells can be differentiated from viable cells based on their differing forward (FSC) and side (SSC) light scattering characteristics (Fig. 1) (Schwartz and Osborne 1995). The frequent sampling provided by automated flow cytometry yields a detailed time course of the total cell count and the population fractions of apoptotic and viable cell populations (Fig. 2A–C). Due to the high sampling frequency, the interconnected dynamics of the viable and apoptotic sub-populations can be precisely determined. On the basis of these data one can clearly differentiate between different growth phases of the culture. Lag phase is defined by an initially decreasing or constant total cell count (arrow a, Fig. 2A2). Lag phase ends when the culture transitions to the proliferation phase, distinguished by an increase in the viable

cell number (arrow a, Fig. 2A2). Proliferation ends when the culture transitions to stationary phase, distinguished by a constant viable cell number (arrows b, c, Fig. 2A1, A2). Subsequently, stationary phase ends when the culture transitions to the death phase distinguished by a decrease in the viable cell number (arrow d, Fig. 2B1).

The culture electroporated in an exponentially proliferating state (culture E) initially showed a lag phase, as evidenced by decreases in the total and viable cell counts and by an increase in the apoptotic cell count resulting in a decrease in the viable cell fraction until approximately 18 h (arrow a, Fig. 2A–D). At this time, the culture started proliferating, and continued increasing in cell number until stationary phase was reached at hour 125 (arrow b, Fig. 2A–D). Interestingly, in culture E, the onset of apoptosis, as indicated by a significant increase in apoptotic cells, preceded the transition to stationary phase by approximately 15 h (Fig. 2C). The onset of apoptosis was likely induced by a nutrient depletion event.

In contrast, the culture electroporated in a stationary state (culture S) initially proliferated as evidenced by increases in the total and viable cell counts, as well as a decrease in the apoptotic cell count resulting in an increase of the viable cell fraction (Fig. 2A, B, D). The end of this initial proliferation was reached at 12 h and is marked by arrow (c). At this time point, apoptotic cells began appearing, and increased at a constant rate for the duration of the culture (arrow c, Fig. 2C). It is interesting to observe two distinct proliferation periods in the total and viable cell counts, with each proliferation phase occurring at a constant rate. The initial proliferation period ends at 12 h (arrow c) and is followed by a slow growth phase that ends at 38 h (arrow d). Towards the end of the second proliferation period, proliferation is evidently balanced by cell death as reflected in the constant fraction of viable cells that is reached during that time. At hour 38, proliferation ceased and the culture entered death phase (arrow d, Fig. 2A, C). Table 1 summarizes the properties of the cultures at the transitions between each growth phase.

Fig. 2 CHO culture growth, viability, and death curves. Culture E (●) and Culture S (○). **(A)** Total cell concentration. Samples were counted every 21 min using the automated flow cytometry setup as previously described (Kacmar and Srienc 2005). The high frequency sampling of total cell number enables determination of distinct growth phases: arrow a indicates the end of lag

phase, arrow b indicates onset of stationary phase, arrow c indicates onset of cell death coupled with growth, and arrow d indicates death phase. **(B)** Viable cell concentration as determined by light scattering properties. **(C)** Apoptotic cell concentration as determined by the light scattering properties. **(D)** The fraction of viable cells

Table 1 Growth characteristics of cultures E and S, denoting transitions between growth phases, initial and final cell counts, and initial and final viabilities, as indicated in Fig. 2

Parameter	Culture E	Culture S
Initial viable cell concentration (cells ml ⁻¹)	2×10^4	8×10^4
Initial viability (%)	66	65
Lag phase exit (h)	18	0
Proliferation rates (h ⁻¹) (before/after fed batch addition)	0.0387/0.0496	0.0453/n/a
Peak viability (%)	99	81
Steady state apoptotic cell concentration (cells ml ⁻¹)	1×10^4	n/a
Onset of apoptosis (h)	110	12
Stationary phase time (h)	125	n/a
Onset of death phase (h)	n/a	38
Final cell concentration (cells ml ⁻¹)	2×10^6	6×10^4
Final viability (%)	97	48

Culture E was inoculated with an electroporated exponentially growing culture, while culture S was inoculated with an electroporated stationary phase culture

Transfection efficiency

After electroporation, cells can only express eGfp if they received plasmid DNA. The viable cells that eventually become fluorescent must have received plasmid DNA during electroporation and are able to express, with a certain delay, the obtained genes. Therefore, the peak of the fraction of fluorescent cells within the viable cell population can be considered an indirect measure of the transfection efficiency. Culture E has a very high transfection efficiency of 60% (arrow b, Fig. 3A). For culture S, the transfection efficiency was only 20% (arrow d, Fig. 3A).

Discontinuities in the slope of the time-course of the fluorescent fraction indicate changes in the rates of apoptosis, proliferation, and plasmid expression between the fluorescent and non-fluorescent sub-populations. For culture E, three discontinuities are apparent (arrows a–c, Fig. 3A). The discontinuity marked by arrow a, Fig. 3A corresponds to the transition of an increasing fluorescent cell concentration to a constant fluorescent cell concentration (arrow a, Fig. 3D). However, while the fluorescent cell

concentration is constant, the non-fluorescent cell concentration continues to decrease until cell proliferation starts (arrow b, Fig. 3A, D). Therefore, it appears that apoptosis was preferentially initiated in the non-fluorescent sub-population. Culture S exhibited a similar initial pattern of a rapid increase followed by a slower increase in the time-course of the fluorescent fraction (arrows d, e, Fig. 3A). In contrast, the cell concentrations of the fluorescent and non-fluorescent sub-populations of culture S exhibited different kinetics for the initial gene expression (arrows d, e, Fig. 3C). There was a rapid increase in the fluorescent cell concentration of culture S until arrow d when a slower rate of increase was observed. For culture S, it is important to note that the non-fluorescent population did not preferentially undergo apoptosis. Therefore, these data indicate that two separate processes, the preferential plasmid uptake (i.e. differing k_T values) and preferential apoptosis induced by the transfection procedure (i.e. differing effective growth rates, μ_F^* and μ_N^*), determine the peak transfection efficiency.

After the peak transfection efficiency of cultures E and S is reached (arrows b, e, Fig. 3A) the fluorescent fraction remains constant. For culture S, one can observe that different effective growth rates (μ_F^* and μ_N^*) are equal after the peak fluorescent fraction is reached. Furthermore, the μ_F^* and μ_N^* values are equal despite differing viable cell proliferation and apoptosis rates (Fig. 2B, C). Therefore, this indicates that after hour 25, apoptosis and proliferation was not preferentially initiated in either the fluorescent or the non-fluorescent cells (Fig. 3C, D). For culture E, the fluorescent fraction began to decrease after the fed-batch addition (arrow c, Fig. 3A). This is due to the non-fluorescent population increasing in number at a statistically significant 14% faster rate than the fluorescent population (Fig. 3D). This trend has two explanations: differing proliferation rates of the fluorescent (μ_F^*) and non-fluorescent sub-populations (μ_N^*) or transitions from a fluorescent to a non-fluorescent cell (negative k_T values). Most likely, cell proliferation has sufficiently diluted eGfp and plasmid to below the detection threshold thus generating a negative k_T value.

Fig. 3 eGfp expression. **(A)** Time course of the fluorescent fraction. Culture E (●) and Culture S (○). For Culture E, the fluorescent fraction dropped due to the different growth rates of fluorescent and non-fluorescent cells. **(B)** Time course of the mean single cell fluorescence of the fluorescent population. Culture E (●) and Culture S (○). **(C)** Growth curves for culture S fluorescent fraction

(○), and culture S non-fluorescent fraction (Δ). **(D)** Growth curves for culture E fluorescent fraction (●), culture E non-fluorescent fraction (▲). For culture E, after the fed-batch addition, the fluorescent cells grew a statistically significant 14% slower than the non-fluorescent cells. Before the fed-batch addition there was not a statistically significant difference in the growth rates

eGfp expression and partitioning during cell division

The physiological state of the cells prior to electroporation had a significant effect on the eGfp expression. The peak single cell eGfp expression of culture E was two times higher than culture S (Fig. 3B). However, after reaching a peak value, the mean single cell fluorescence of culture E decreased exponentially, while in culture S, the mean single cell fluorescence remained constant. This indicates that the production and degradation of eGfp are balanced in the non-proliferating culture S. Furthermore, as seen in culture E, dilution effects due to cell proliferation can perturb the balance between synthesis and degrada-

tion of eGfp. This appears to be the case since culture E proliferated, and the non-replicating plasmid and expressed eGfp were partitioned between daughter cells. The partitioning therefore leads to an exponential decrease in the mean single cell fluorescence of culture E. In contrast, culture S did not proliferate and thus partitioning did not occur. Therefore, a constant mean single cell fluorescence of the fluorescent fraction was observed.

Examining the time-course of histograms representing single cell green fluorescence of culture E, reveals an interesting effect caused most likely by the partitioning of plasmid and eGfp (Fig. 4). In this graph one can clearly recognize five sub-populations with different,

Fig. 4 Single cell distribution of eGfp fluorescence for the exponentially growing culture. The histogram is normalized to the maximum value of the distribution. Note the distinct, discrete sub-populations that evolve after peak fluorescence is reached

discrete levels of eGfp content. Initially, cells are charged with a large amount of plasmid and subsequently express eGfp at the highest discrete level of eGfp content. As cell division occurs, eGfp and plasmid is partitioned between daughter cells and the next discrete level of eGfp content appears. This process continues until a cell dilutes its plasmid and eGfp content to the lowest discrete sub-population, and is then termed non-fluorescent. Therefore, each discrete level of eGfp content corresponds to a generation of cells.

Total eGfp production

Multiplication of the fluorescent cell number concentration in the bioreactor with the average single cell fluorescence results in the total eGfp content in the culture at each time point. Total eGfp production in culture E increases linearly with time until 15 h before stationary phase is reached at hour 110 (Fig. 5). The linear rate of eGfp accumulation in the culture may indicate that proliferating cells are able to maintain the initial plasmid copy number. Since the plasmids are not replicated their total number presumably remains constant resulting in a constant rate of eGfp expression. This would indicate that plasmids are not significantly degraded during cell proliferation. Past 110 h cultivation time, the onset of apoptosis occurs (arrow b, Fig. 2C, arrow a, Fig. 5)). Subsequently, as transfected cells undergo apoptosis, plasmid and eGfp are lost from the viable cell population. Therefore, apoptosis

Fig. 5 Total eGfp production for Culture E (●) and Culture S (○). Total eGfp production is defined by multiplying the total fluorescent cell number in the bioreactor by the average single cell fluorescence at each time point

artificially decreases the rate of total eGfp production, and makes it appear that plasmid has been exhausted. A similar effect is seen in culture S, since the slope of the total production lines change at hours 12 and 32, which corresponds to the onset of apoptosis (arrow c, Fig. 2C) and the termination of proliferation (arrow d, Fig. 2B).

Cell size and intracellular structure

Further insight into the state of the culture is given by the light scattering measurements. The small angle light scattering of a cell (FSC) correlates with cell diameter. Since electroporation opens holes in the cell membrane, cell mass is lost to the supernatant. After electroporation, the cellular membrane reseals and these cells are detected as the viable fraction. Before proliferation begins, cells in early G1 must increase their mass. Therefore, cultures E and S increase their mean cell size immediately before proliferation is initiated. The initial increases in cell size of cultures E and S occurs linearly. For culture E, at hour 18, cell proliferation began and there was a corresponding decrease in cell size (Figs. 6A, 7). The increase in FSC at hour 10 for culture E corresponds to the increase in the SSC at the same time point (Fig. 6C). Since SSC correlates with the density of internal cellular structures, this indicates that cell growth also significantly affects the internal cell structure.

Fig. 6 Cell size and cell granularity. Culture E (●) and Culture S (○). **(A)** Average single cell size. Arrow indicates time of fed-batch addition to exponentially

growing culture. **(B)** CV of cell size. **(C)** SSC vs time. Arrow indicates time of fed-batch addition to exponentially growing culture. **(D)** Time course of CV of SSC

The next two peaks in cell size of culture E occur with a period of 18 h. These regular peaks can be likely explained by a partial synchrony in

Fig. 7 Cell size. Time course histogram of the forward scatter for culture E. The histogram is normalized to the maximum value of the distribution

the culture, since the doubling time during this period was approximately 18 h. At hour 72, the fed batch addition to culture E caused a change in the osmolality of the culture. Thus, the mean FSC increased, and then returned over 10 h to the previous level (Fig. 6A, arrow). The increase in the cell size of culture E at hour 72 is mirrored by a decrease in SSC (Fig. 6C, arrow).

For culture S, two time periods can be distinguished on the basis of observed changes in cell size. The first time period is the immediate recovery from electroporation that occurs during hours 0–12. The second time period occurs from 12 to 80 h when FSC increases linearly (Fig. 6). This corresponds with a linear increase in the SSC of culture S (Fig. 6C). Surprisingly, despite increases in the cell size and the internal complexity of cells the cell number does not increase and also, the single cell eGfp content remains constant.

This is in direct contrast to culture E, where an increase in cell size and cell granularity preceded the beginning of cell proliferation.

Discussion

The detailed observation of the population dynamics of transient gene expression yields several interesting observations. The data clearly shows that (i) preferential plasmid uptake and preferential cell death between transfected and non-transfected populations determine the transfection efficiency; (ii) subsequent generations of proliferating transfected cells have distinctly different protein contents resulting in discrete subpopulations representing each cell generation; (iii) total eGfp production increases linearly with time; (iv) two fundamentally different stationary and death phases can be reached. One type of stationary phase occurs when proliferation ceases while cells decrease their cell size, maintain granularity, and mean eGfp content decreases (Culture E, hour 120+). The second type of stationary phase occurs when proliferation ceases while cells increase their cell size, increase granularity, and mean eGfp content is constant (Culture S).

The transfection process is affected by two separate processes including preferential cell death and preferential plasmid uptake. These two processes are dependent on both the physiological state of the cells prior to transfection and the parameters of the transfection procedure. Previous work has shown that cell size (Valic et al. 2003), cell cycle position (Golzio et al. 2002), and membrane tension (Barrau et al. 2004) are physiological variables that affect the transfection efficiency for electroporation. Also, the voltage gradient, pulse duration, and pulse shape are transfection parameters that affect the transfection efficiency for electroporation (Valic et al. 2003). However, each of these studies only examines the peak transfection efficiency, and not the separate processes of preferential cell death and plasmid uptake that the culture underwent to attain the peak transfection efficiency. This is an important observation since two separate processes influence the transfection efficiency, and thus identical transfection efficiencies can be

obtained for differing final physiological states of the culture. Therefore, variations in culture performance and final product quality would be observed.

The observation of a linear increase in total eGfp content coupled with the observation of discrete levels of single cell protein content indicate that cell proliferation is irrelevant to the production rate of the desired product. This is because it is likely that plasmid is conserved between subsequent generations of cells. Therefore, at all times, a constant level of plasmid is being transcribed, and thus a constant amount of desired product is produced. Similar linear production rates of desired protein in a non-replicating plasmid system are seen for different transfection techniques (Derouazi et al. 2004). Therefore, to attain maximum production, either the onset of apoptosis should be avoided or the proliferation of the culture should be arrested. Furthermore, the observation of a linear increase in total eGfp content yields a quantitative description of the average single cell eGfp content as described by Eq. 6. Equation 6 accurately predicts the time course of the single cell protein content as shown for culture E in Fig. 3C. However, Eq. 6 does not describe the average single cell eGfp content observed for culture S. It is likely that different culture physiology between cultures E and S are responsible for the different observed expression kinetics. These differences are highlighted by the two different stationary phases that were observed.

The observation of two distinct stationary phases provide qualitative evidence supporting the hypothesis that cell size increase and cell proliferation are two independent processes in mammalian cell culture (Conlon and Raff 2003).

Conclusions

The automated flow cytometry system provides a unique capability to study the time course of cell phenotype in unprecedented detail. New variables like cell counts, cell size, cell granularity, protein expression, as well as virtually any phenotype that has a cytometry stain can now be examined on-line. This opens up new and unique

capabilities to understand cell physiology and to implement new and unique process control strategies. The described approach yields valuable insight into how cultures respond to specific treatments that are relevant to industrial processes and operations.

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