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A quantitative method for the analysis of mammalian cell proliferation in culture in terms of dividing and non-dividing cells

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Abstract. The application of the exponential growth equation is the standard method employed in the quantitative analyses of mammalian cell proliferation in culture. This method is based on the implicit assumption that, within a cell population under study, all division events give rise to daughter cells that always divide. When a cell population does not adhere to this assumption, use of the exponential growth equation leads to errors in the determination of both population doubling time and cell generation time. We have derived a more general growth equation that defines cell growth in terms of the dividing fraction of daughter cells. This equation can account for population growth kinetics that derive from the generation of both dividing and non-dividing cells. As such, it provides a sensitive method for detecting non-exponential division dynamics. In addition, this equation can be used to determine when it is appropriate to use the standard exponential growth equation for the estimation of doubling time and generation time.

Often in mammalian cell culture studies, there is a need to quantitate the proliferative capacity of cells. This need arises both in the analysis of the intrinsic growth properties of cells and in the investigation of changes in such properties due to effects in the extracellular and intracellular environment. By far, the method most commonly used for such studies is the estimation of population doubling time (DT) by application of the exponential growth equation (EGE), $N = N_0 e^{kt}$. When cells in a growing population accumulate according to this equation, a value for the constant k can be determined from the slope of linear plots of ln (N) versus time. The DT for such a cell population can be calculated using the equation, $\ln(2) = k(DT)$. This method has become standard for the quantitation of mammalian cell proliferation in culture.

The widespread use of the EGE is a testament to its general applicability to the growth of diverse cell types in culture. This popularity also leads to its occasional misuse. Implicit in the application of the EGE is the assumption that all cells in the studied population divide to give rise to two daughter cells that also both divide. Thus, the fraction of daughter cells that divide (herein designated as F_d) is assumed to be 1.0. In this ideal case, the DT is equivalent to the generation time (GT) of individual cells in the population (Steel 1968). Unfortunately, this highly useful

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approximation is often taken for granted in mammalian cell culture studies. For cell populations with an F_d that is significantly less than 1.0, DT is no longer a constant, but instead increases with time. When the EGE is inappropriately applied to such cell populations, the DT determined is really an average value for the studied period of growth. While such DT values are still useful in comparisons for relative growth rate, they lead to serious errors in the estimation of GT. Such errors can lead to critical misinterpretations of the cell division dynamics that underlie the proliferative properties of cells in culture.

While cells in culture are routinely characterized by exponential growth kinetics, this type of cell growth is unusual *in vivo*. The predominant form of cell division in mature animal tissues is stem cell renewal division (Thrasher 1966, Cairns 1975). Unlike exponential growth, which is symmetric with respect to the division of daughter cells, renewal growth is asymmetric. Dividing stem cells in somatic tissues give rise to two daughter cells that are asymmetric in their capacity for division. One daughter retains the division potential of the mother cell, while the other is a non-dividing cell that differentiates to become a functional constituent of the tissue. The F_d of such a division program is 0.5. Were this type of cell growth maintained in cell culture, it would give rise to non-exponential, linear population growth kinetics. This is an excellent example of a situation in which the application of the EGE would give a false impression of an important biological process. In error, it would dictate an exponentially growing population with an unusually long GT.

In a recent study of the growth properties of cells that have experimentally elevated levels of the *p53* antioncogene product, we observed linear population growth kinetics that were due to stem cell division kinetics in cell culture (Sherley, Stadler & Johnson 1995). In the course of the same study, we found that two human primary cell lines also exhibited non-exponential population growth kinetics. Thus, the EGE was inappropriate for the growth analysis of these cell types. In this report, we describe the derivation of a population growth equation that defines cell growth in terms of the variable F_d, the fraction of daughter cells that divide. This equation is more general in its derivation than the EGE, and thus has broader application to the growth of cells in culture. Unlike the EGE, it allows the quantitative assessment of populations of cells that grow with either exponential or non-exponential division kinetics. In this report, we show that application of this equation can lead to the detection of previously overlooked division properties of particular cell types in culture.

MATERIALS AND METHODS

Cell culture

Immortalized control and p53-inducible cell lines were maintained as previously described (Sherley 1991). Human primary cells were cultured as recommended by the American Type Culture Collection (ATCC) and were analysed at one passage after thawing from the original ATCC cryovials.

Population growth analyses

Previously, we have described the application of microcolony assays to the study of cell division dynamics in culture and to the determination of GT (referred to as T_{cc} in previous reports; Sherley *et al.* 1995). Data from such analyses were used to construct population growth curves. At each time point in a microcolony analysis, the total number of cells present was calculated by: **1** multiplying the observed number of microcolonies by the number of cells that is comprised; and **2** summing the products.

For the standard population growth analysis with control and p53-inducible lines, the cells were first grown for 48 h under non-inducing conditions (37°C) to 1/8 to 1/4 confluency in Dulbecco's modified Eagle medium supplemented with 10% dialyzed fetal bovine serum (JRH Biosciences) and 1 mg/ml G418 sulfate (Gibco Laboratories). They were then replenished with fresh media and, 16 to 24 h later, trypsinized, diluted to the required density, and replated at 37°C in 75 cm^2 culture flasks. After 24 h at 37°C, the replated cell cultures were placed at 32.5°C, thus initiating the analysis by inducing the low temperature-dependent expression of p53 (Sherley 1991). Thereafter, at the indicated times, the cells were trypsinized and counted using a model ZM Coulter Counter.

Mathematic derivation

For the derivation of an equation that defined cell growth in terms of the fractions of dividing and non-dividing cells we began by considering the following general cell division progression,

t/GT	*N*
0	N_0
1	$(1-F_d)N_0+2F_dN_0$
2	$(1-F_d)N_0+(1-F_d)(2F_dN_0)+2F_d(2F_dN_0)$

where t = time; N = the number of cells at time t; N_0 = the initial number of cells; GT = the length of time for one cell cycle; and F_d = the dividing fraction of newly divided daughter cells. In this progression, F_d defines the fraction of dividing cells, and $1-F_d$ defines the fraction of non-dividing cells. The summation of this progression can be written at $t/GT = k$, where k is an integer, as:

$$N = N_0\left[1/2 + 1/2\sum_{i=0}^{k}(2F_d)^i\right]$$

Applying the identity:

$$a\sum_{i=0}^{n-1} x^i = a[(1-x^n)/(1-x)]$$

where $n-1=k$; $x=2F_d$; and $a=1/2$, this summation can be written as:

$$N = N_0\left[1/2 + \frac{1-(2F_d)^{k+1}}{2(1-2F_d)}\right]$$

Letting $k = t/GT$ and interpolating between integral values of t/GT,

$$N = N_0\left[1/2 + \frac{1-(2F_d)^{(t/GT+1)}}{2(1-2F_d)}\right] \quad (1)$$

Determination of F_d

For F_d determinations, triplicate population growth data were averaged, and their means were plotted relative to the initial cell number, N_0. The software Macintosh Matlab, version 3.5 (The Mathworks, Inc, MA, USA) was used to calculate a series of curves for equation (1) by varying F_d

while holding constant the experimentally determined input values of N_0 and GT. Non-linear least squares regression analysis with the Levenberg–Marquardt algorithm (Bard 1974) was used to determine the curve that gave the best fit to each data set. The F_d value that corresponded to the curve that best fit the data was designated as the F_d of the analysed cell population.

Statistical analyses

The level of statistical significance of all growth data regression fits was determined from standard tables for correlation coefficients (r^2 values) versus degrees of freedom.

RESULTS

We developed a mathematical method to quantify the proliferative character of cells in terms of a single parameter, F_d. F_d is defined as a fraction of daughter cells that divide. Based on a cell growth progression series that incorporated the idea of the generation of both dividing and non-dividing daughter cells in a growing population, we derived equation (1) (see Materials and Methods above for details of derivation).

$$N = N_0 \left[1/2 + \frac{1 - (2F_d)^{(t/GT+1)}}{2(1-2F_d)} \right] \quad (1)$$

Equation 1 defines the number of cells (N) in a growing population at any time (t) in terms of the three variables, N_0, GT and F_d. When N_0 and GT are known, equation (1) can be used to determine the value of F_d from simple population growth data.

Figure 1I depicts the series of growth curves defined by equation (1) for different values of F_d. When F_d equals 1.0, equation (1) simplifies to the form of the simple exponential growth equation:

$$N = N_0 e^{kt}$$

where $k = \ln 2/GT$. Thus, an F_d value of 1.0 corresponds to an ideal exponential growth curve. For F_d values that are less than 1.0 but greater than 0.5, curves with less than the ideal, but still basically exponential kinetics are described. For values less than 0.5, asymptotic curves are defined. The value of 0.5 for F_d is a special case. When F_d equals 0.5, equation (1) simplifies to:

$$N = N_0(1/2 + 0/0)$$

which is an indeterminate form that must be evaluated using L'Hospital's Rule. Namely,

$$\begin{aligned} N &= \lim_{F_d \to 1/2} N_0\{1/2 + [1 - (2F_d)^{t/GT+1}/2(1-2F_d)]\} \\ &= N_0/2 + \lim_{F_d \to 1/2} N_0\{(d/dF_d)[1 - (2F_d)^{t/GT+1}]/(d/dF_d)[2(1-2F_d)]\} \\ &= N_0/2 + \lim_{F_d \to 1/2} N_0\{[(2F_d)^{t/GT+1}](t/GT+1)(1/F_d)/4\} \\ &= N_0/2 + (N_0/2)(t/GT+1) \\ &= (N_0/2GT)t + N_0 \end{aligned}$$

Thus, for F_d equals 0.5, equation (1) defines a line of the simple form:

$$y = mx + b$$

where m, the slope, equals $N_0/2GT$ and the y-intercept b equals N_0.

Equation 1 was applied in the analysis of three cell types with distinctive growth properties in culture. Line 1g-1 is a G418-resistant derivative of the immortalized murine mammary epithelial line C127. This line, and others like it, was derived as a control for the expression elements used to construct cell lines that contain inducible *p53* genes. These cells exhibit typical exponential division kinetics. Line 1h-3 is a G418-resistant, p53-inducible derivative of C127 cells. When grown under conditions that induce p53 expression (32.5°C), these cells switch from exponential division kinetics to stem cell division kinetics (Sherley 1991, Sherley *et al.* 1995). Two human primary cell lines were analysed. WI-38 is a lung fibroblast line (Hayflick 1965) and Fhs74Int is an intestinal epithelial line (Owens *et al.* 1976). Both primary cell lines exhibit senescence in cell culture (Hayflick 1965, Hay *et al.* 1992).

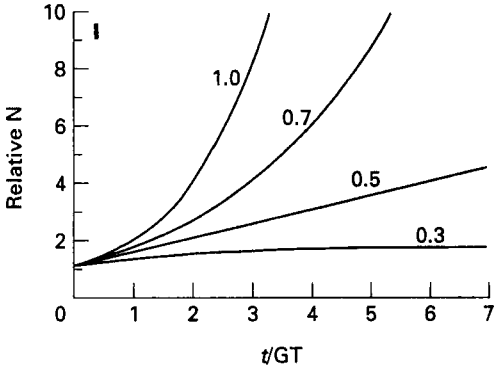
The determination of F_d requires an independent measure of GT. We have described a simple method for the determination of GT for adherent cell cultures (Sherley *et al.* 1995). In brief, serial microscopy examinations are used to determine the average time required for cells in microcolonies to divide. Others have described methods for the determination of GT that are applicable to either adherent or suspension cultures (Sisken 1964, White, Terry & Meistrich 1990, White *et al.* 1991). Initially, equation (1) was applied to population growth data constructed from microcolony analyses (Figure 1). In this case, the input value for GT was determined at the same time that the growth data were collected. The same value of GT was applied to population growth data derived at substantially increased cell densities (Figure 2).

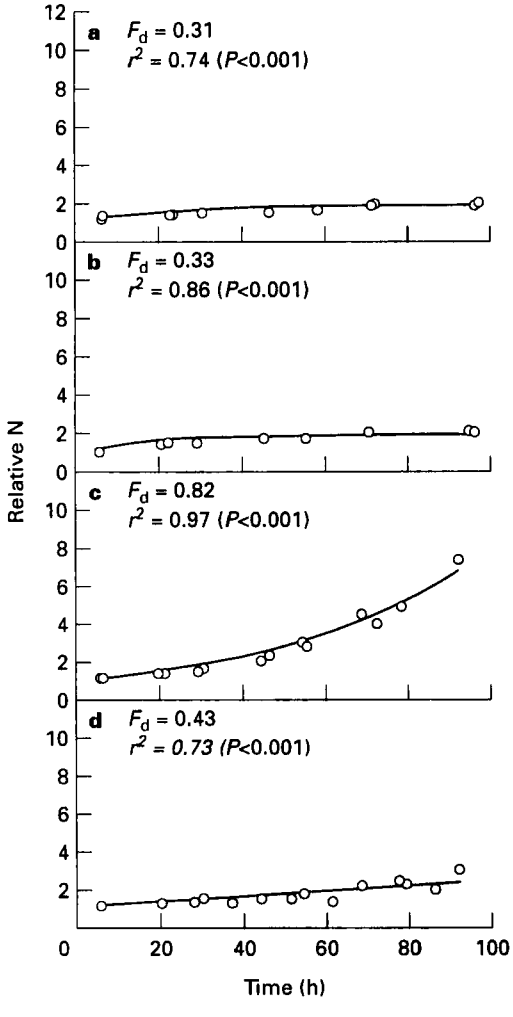
Figures 1 and 2 show examples of F_d determinations for each of the three cell types. The F_d value determined in each case was consistent with the unique division dynamics of the respective cell type. The value of F_d determined for the immortalized control cells was 0.99 ± 0.18 (Mean $\pm$ SD; $n = 6$), indicating typical exponential division kinetics. The mean value of F_d for the primary cells was 0.32 ($n = 2$). This value of F_d is consistent with the growth senescence behaviour of these cells in culture (Hayflick 1965, Hay *et al.* 1992). However, it indicates that the senescence behaviour of these cells is not due to an increase in GT, as previously held (Hayflick 1965); but instead is due to the production of non-dividing cells at a rate that exceeds the production of dividing cells. The value of F_d determined for p53-induced cells was 0.51 ± 0.08 (Mean $\pm$ SD; $n = 4$), indicating a stem cell division pattern in which the production of dividing cells is balanced by the production of non-dividing cells.

DISCUSSION

These results with primary cells and cells with elevated wildtype p53 expression serve to highlight the unique advantage of the application of equation (1) in cell growth analyses. In earlier studies, changes in the growth of both these type of cells has been attributed to processes that uniformly affect the GT of all cells in the population. We have demonstrated that this is not the case (Sherley *et al.* 1995) and that, in fact, changes in the population kinetics of such cells reflect cellular programs that give rise to non-dividing cells while maintaining the GT of dividing cells. It seems likely that other cell populations, when appropriately analysed, will yield similar findings. Application of equation (1) to population growth data provides a sensitive method for the detection of non-exponential division kinetics; and the determined value of F_d can be an important clue to the nature of the underlying cell division dynamics.

The mathematical treatment of proliferating cell populations with division fractions less than 1.0 has been considered previously by Steel (1968). Using similar assumptions, Steel derived an equation (equation 7, in Steel 1968) that defined the DT of proliferative tumour cell populations in terms of the GT of dividing cells and a constant, α, the percentage of proliferative cells produced at each division. The percentage of non-dividing cells produced at each division is given


Relative N
t/GT
1.0
0.7
0.5
0.3


a F_d = 0.31
r^2 = 0.74 (P<0.001)
b F_d = 0.33
r^2 = 0.86 (P<0.001)
c F_d = 0.82
r^2 = 0.97 (P<0.001)
d F_d = 0.43
r^2 = 0.73 (P<0.001)
Relative N
Time (h)

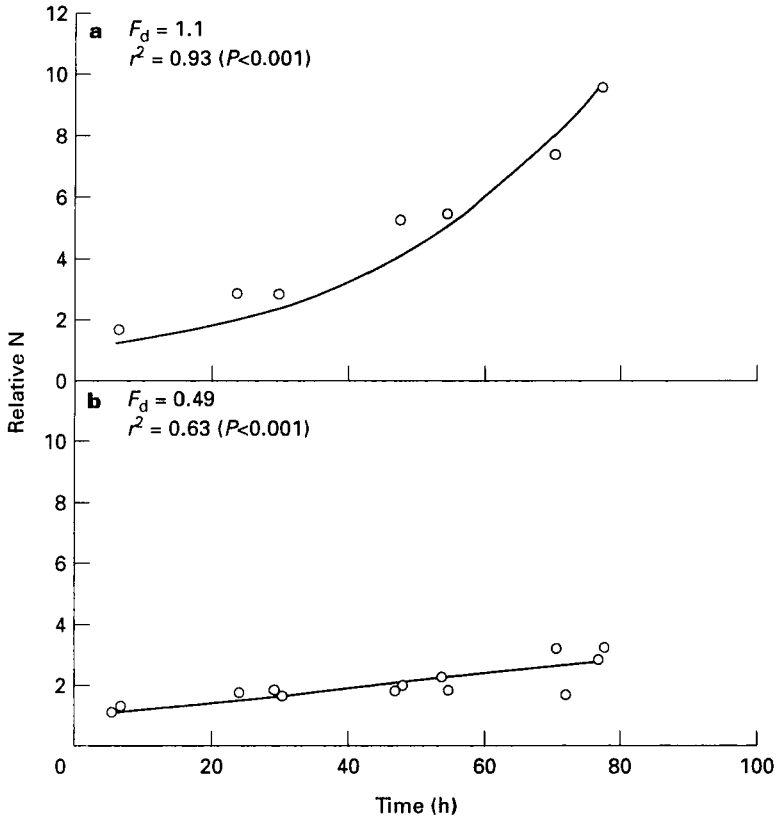


Figure 2. F_d determinations for populations of control and p53-induced cells at higher cell density. At the indicated times, the number of cells in each growing population was determined as described in Materials and Methods. The data were plotted relative to the initial cell number (N_0) and fit to equation (1) to determine F_d. The average GT determined for each cell line in microcolony analyses was used (see Figure 1 caption). **a** Data for control 1g-1 cells ($N_0 = 1.0 \times 10^4$ cells per 75 cm^2; GT = 26 h). **b** Combined data from two experiments for p53-induced 1h-3 cells ($N_0 = 0.5 \times 10^4$ and 0.7×10^4 per 75 cm^2; GT = 21 h).

Figure 1. F_d determinations with microcolony growth data from populations of primary cells, immortalized control cells, and p53-induced cells. (**I**) Graph of the family of curves describing the growth of cells in culture as predicted by equation (1) (see Materials and Methods) for different values of the variable F_d, the fraction of daughter cells that divide. Values greater than 0.5 correspond to exponential growth, with 1.0 being the ideal; values less than 0.5 describe senescing populations; and a value of 0.5 predicts linear population growth kinetics. **a–d** Data from microcolony progression analyses were used to construct population growth curves. The cell numbers (N) at the indicated times were plotted relative to the initial cell number (N_0) and fit to equation (1) to determine F_d as detailed in Materials and Methods. The GTs required for the analysis were determined independently from each set of microcolony data. Growth data were combined for replicate experiments and, therefore, averaged GT values were used for each determination. **a** WI-38 diploid lung fibroblasts at passage 14 (duplicate data sets; $N_0 = 300$ per 75 cm^2; average GT = 9.1 h). **b** Fhs74Int diploid intestinal epithelial cells at passage 25 (duplicate data sets; $N_0 = 300$ per 75 cm^2; average GT = 12 h). **c** Control 1g-1 cells (duplicate data sets; $N_0 = 140$ and 400 per 75 cm^2; average GT = 26 h). **d** p53-induced 1h-3 cells (triplicate data sets; $N_0 = 140$, 160 and 400 per 75 cm^2; average GT = 21 h).

by $2-\alpha$. In our treatment, F_d is equivalent to $\alpha - 1$. Our analysis of F_d assumes that the lifetimes of dividing cells are constant and equal to GT. Based on experimental data, this approximation is a reasonable one for the growth of cell cultures, which are typically homogeneous for cell type. In direct analyses of the division of single cells in cultures of control and p53-induced cells, which vary greatly for F_d, we found that lifetime measurements show only about a 20% standard deviation from their means (Sherley *et al.* 1995).

While Steel's treatment is a first demonstration that, in the setting of significant production of non-dividing cells, DT will be greater than GT, it does not provide equations that can be straightforwardly applied to population growth data from cells in culture. Our independent derivation of equation (1) provides such a research tool. In addition to its use in determining F_d for a population for which GT is known from independent analyses, equation (1) can also be used to determine when it is appropriate to use the EGE as a method to estimate the DT and GT for a cell population. The GT estimated by the EGE method can be input along with N_0. F_d can then be determined by fitting equation (1) to the experimental growth data. If the determined value of F_d is not close to 1.0, then it is very likely that the EGE has been improperly used and that the examined population is not growing with ideally exponential kinetics. Depending on how far the experimentally determined value of F_d differs from 1.0, additional analyses may be indicated. In this way the application of equation (1) in studies of mammalian cell proliferation can limit errors due to improper use of the EGE; and thus lead to the discovery of unique cell division dynamics in culture that otherwise would go unnoticed.

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