Special Feature

Analysis of growth kinetics by division tracking

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Abstract Cell division tracking using fluorescent dyes, such as carboxyfluorescein diacetate succinimidyl ester, provides a unique opportunity for analysis of cell growth kinetics. The present review article presents new methods for enhancing resolution of division tracking data as well as derivation of quantities that characterize growth from time-series data. These include the average time between successive divisions, the proportion of cells that survive and the proliferation per division. The physical significance of these measured quantities is interpreted by formulation of a two-compartment model of cell cycle transit characterized by stochastic and deterministic cell residence times, respectively. The model confirmed that survival is directly related to the proportion of cells that enter the next cell generation. The proportion of time that cells reside in the stochastic compartment is directly related to the proliferation per generation. This form of analysis provides a starting point for more sophisticated physical and biochemical models of cell cycle regulation.

Key words: carboxyfluorescein diacetate succinimidyl ester, cell cycle analysis, cell division tracking.

Introduction

Cell division tracking is a powerful adjunct to DNA analysis, because it defines the time when cells divide and how many divisions they have undergone since labelling. An important prerequisite for accurate measurement of divisional history is the development of methodology that resolves successive cell generations as distinct clusters. For many cell types staining with fluorescent dyes, such as carboxyfluorescein diacetate succinimidyl ester (CFSE), is non-homogeneous, without resolution of distinct cell generations. The present article will review a methodology that increases the resolution of fluorescence division tracking so that even non-homogeneously stained cell types can be studied. We will also develop methods for analysis of growth kinetics, specifically focusing on generation time, cell proliferation and survival.

High resolution division tracking

Lyons and Parish discovered fluorescent cell division tracking. They observed distinct clusters on CFSE histograms that corresponded to consecutive lymphocyte divisions that had occurred *in vitro* or *in vivo*.¹ The mean fluorescence of each cluster decreased geometrically by a factor of two and appeared as uniformly spaced clusters on a log scale. The resolution of divisional clusters was related to the initial staining. Other dyes, such as PKH26, did not stain cells as uniformly as CFSE and divisional clusters were not resolved.²

Consecutive generations are easily distinguished if the initial staining of cells is confined to a two-fold range of

fluorescence. It was demonstrated that haematopoietic cells, which stained over a wide range of fluorescence, could be tracked with high resolution by sorting a subpopulation with homogenous fluorescence.³ The channel width for sorting cells that do not vary more than two-fold in fluorescence intensity is calculated as follows:

Width of gate (channels)
$$< \log_{10}(2) \frac{\text{total channels}}{\text{total decades}}$$
 [1]

Typically, the width of the sort window using a fourdecade logarithmic amplifier displaying binned data in 256 channels should be less than 19 channels. In practice, depending on the coefficient of variation (CV) of the sorter, a proportion of the sorted cells will have fluorescence values outside of the sort region and a narrower sorting gate than the theoretical value described above is required. The CV is measured by sorting fluorescent beads from a single channel (fluorescein isothiocyanate beads for CFSE, phycoerythrin beads for PKH26) and reanalysing the sorted population. We have obtained best results using CFSE and a 10-channel sort window. Figure 1 shows the resolution of separate divisions using a sorted- versus unsorted-cell line.

This sorting technique has been successfully applied to a variety of primary haematopoietic cells, including fresh and cryopreserved bone marrow, cord blood and mobilized peripheral blood. By combining this sort gate with others, the divisional recruitment of haematopoietic cells in response to growth factors at any stage of differentiation can be studied.³

We would also like to comment on a few additional technical aspects of this procedure. Most fluorescent tracking dyes leach from cells and CFSE is no exception. Esterase cleavage of CFSE results in formation of a fluorescent polar molecule that is transiently retained inside the cell. In our experience, the reaction of the succinimidyl moiety with intracellular amines is incomplete and an overnight incubation at 37°C is

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Figure 1 Histograms of carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescence obtained for U937 cells labelled with 0.5 µmol/L CFSE and then cultured for 24 h prior to analysis. (a) Following staining, (b) unsorted cells 24 h after staining, (c) following staining and sorting using a 10/256 gate, (d) sorted cells 24 h after staining.

required to remove most of the unbound intracellular dye before sorting. Initially, there is a decrease in fluorescence, which is the result of dye leaching and not cell division. Experimental controls are required to correctly assign generation numbers to each cluster (see later).

The number of successive divisions that can be tracked depends on the fluorescent intensity of stained cells. As the signal decreases, background fluorescence becomes significant and is a noisy variable that is added to the dye signal. Thus, the clusters become broader at low signal levels. Six successive divisions can be resolved provided that the staining intensity is at least 2⁸ times greater than background. This would correspond to stained cells having fluorescence in the fourth decade with background falling within the first logarithmic decade. Because all dyes are toxic at high concentration, some titration is required to obtain optimal staining.

Sorting a narrow window of CFSE fluorescence reduces the cell yield, which is a significant consideration when studying rare cell types. Yield can be increased by sorting cells from two adjacent gates using two-way sorters (left and right sort), although cells collected from these sort regions must be grown in separate cultures.

The CFSE sort region can also affect proliferative behaviour or phenotype. Often neighbouring CFSE gates differ in divisional recruitment kinetics. This is most likely related to selection of different phenotypes or stages of the cell cycle by the sorting procedure. For example intracellular protein and esterase levels are lower in quiescent haematopoietic cells as well as cells that have just divided. This correlates with a lower CFSE staining intensity. Therefore, it is important to demonstrate that the CFSE sort gate does not alter the experimental hypothesis and conclusion.

Assignment of generation number

Correct assignment of generation number to divisional clusters requires the inclusion of an experimental control to measure the rate of dye leakage over the duration of the experiment. Needless to say, it is important to use the same instrument settings for each analysis. In our experience, fixed cells lose dye at a different rate to cells grown in culture and are not useful for identification of undivided cells. The best control is a viable quiescent (non-dividing) cell type that will persist in culture for the duration of the experiment. This is usually not a problem when studying the divisional recruitment of primitive haematopoietic cells, because the stem cell compartment is initially quiescent. A control culture can also contain a cell cycle blocking drug, such as colcimid, although Figure 2 Gating strategy used to enumerate cells in consecutive generations. Magnetic activated cell sorter-enriched CD34+ human umbilical cord blood cells were stained with 1.5 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE), incubated overnight in Iscove's modified Dulbecco medium/10% FCS and stained the next morning with anti-CD34-PE-Cy5 before sorting. Cells were analysed after 2 days in culture in serum-free media containing stem cell factor, megakaryocyte growth and development factor, flt-3L and IL-3, all at 20 ng/mL. (a) Forward (FSC) versus side light scatter (SSC) showing that Coulter bead standards are easily distinguished from viable cells. (b) The CFSE histograms: M1, undivided cells; M2, first division; etc. Gates are 19/256 channels wide.



cells will die after a few days of culture. Often daily serial measurements make it possible to infer generation number by lining up histograms.

Analysis of histogram data

The growth quantities that can be measured from a time series of cell tracking histograms include (i) the frequency distribution of cells in successive generations; (ii) the average number of cell generations; (iii) the average cell cycle duration; (iv) the recovery of progenitors (survival); and (v) proliferation. These quantities and their method of calculation will be defined below.

Absolute cell counts

Absolute cell counts are required for the measurement of cell growth and survival. Manual counting methods are inaccurate, so we have adopted a flow cytometric technique. Known numbers of polystyrene beads are added to the culture (internal standard) and are easily distinguished from cells using forward- and side-scatter parameters (Fig. 2a). The absolute cell count is calculated from the ratio of the number of cells to beads multiplied by the absolute bead count.

[2]
Absolute cell count =
$$\frac{\text{gated cell event}}{\text{gated bead event}} \times \text{absolute bead count}$$

The number of cells in each generation is determined using the gating strategy shown in Fig. 2b.

Progenitors

Progenitors are defined as precursor cells that give rise to progeny during the tracking experiment. With each successive division, two daughter cells are generated. Thus, the number of progenitors is calculated by dividing the number of cells in generation *i* by 2^{i-1} , where undivided cells are the first generation. The number of progenitors derived from the *i*th generation is close to the number of clones that give rise to colonies with 2^{i-1} progeny cells. Calculation of progenitor cell numbers is a useful concept when estimating cell survival, because progenitors are conserved if there is no apoptosis.

The progenitor number giving rise to cells in the *i*th generation at time t, $P_i(t)$, is defined as

$$P_i(t) = N_i(t)/2^{i-1}$$
[3]

where $N_i(t)$ is the number of cells in generation *i* at time *t*.

The frequency distribution of progenitors across successive generations, $f_i(t)$, is estimated by counting the number of progenitors in each generation and dividing by the total number of viable progenitors at time *t* after labelling.

$$f_i(t) = P_i(t) / \sum_j P_j(t)$$
[4]

The summation sign indicates the total number of progenitors from all generations that are viable at time *t*.

$$m(t) = \sum_{i} i f_t(i)$$
 [5]

This distribution is then used to calculate the expected number (arithmetic mean) of the cell generation at time *t*: The average cell cycle duration, $\mu(t)$, is calculated by dividing an increment in time, Δt , by the increase in the number of divisions.

$$\mu(t) = \lim \Delta t \to 0 \frac{\Delta t}{\Delta \text{ average number of divisions}} = 1/m'(t)$$
[6]

where m'(t) is the time derivative of m(t).

The mean cell cycle duration is estimated by plotting the mean generation number with respect to time. Most often



Figure 3 Analysis of growth kinetics. NALM-6 cells were labelled with 2 µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE), cultured in Roswell Park Memorial Institute medium (RPMI) with 10% FCS overnight, sorted (10/256 gate) and then cultured for up to 5 days in RPMI and 10% FCS. Mean generation number (O), proliferation (Δ), and progenitor survival (\bullet) are plotted with respect to time (xaxis). The left y axis shows mean generation number on a linear scale. The right v axis shows progenitor survival and proliferation on a log₁₀ transformed scale. Data points are the mean \pm SD of four cultures. Linear regression is performed on data points following an initial lag period (17 h).

there is a lag before m(t) increases in a linear fashion (Fig. 3). The cell cycle duration is estimated for either progeny or progenitors from the reciprocal of the gradient of this function, as determined by linear regression analysis.

Survival and proliferation

Progenitor survival, s(t), is calculated by summing the number of progenitors in each generation and dividing by the number of cells at the start of the experiment, N_0 .

The total cell expansion is defined as the number of cells

$$s(t) = \frac{\sum_{j} P_{j}(t)}{N_{o}}$$
[7]

generated divided by the input cell number. Net cell growth is determined by the relative rates of prolif-

$$\mathbf{e}(t) = \frac{\sum_{j=1}^{N} N_{j}(t)}{N_{0}}$$
[8]

eration and cell survival. For example, if only 50% of cells survive with each successive generation, the cell number will remain constant, despite the presence of a proliferative process. A third quantity, proliferation, p(t), is a measure of the proliferative process independent of cell death and is defined as follows:

$$p(t) = \frac{\sum_{j=1}^{N_j(t)} p_{j}(t)}{\sum_{j=1}^{N_j(t)} p_{rogenitors}} \qquad [9]$$

If there is no cell death, proliferation is equal to expansion. It is also noteworthy that the absolute cell number is not required for estimation of proliferation, p(t) and the mean generation number, m(t).

Modelling of cell cycle transit

A mathematical model of cell cycle transit provides a physical interpretation for cell division tracking data. Some of the processes that interest biologists are the biochemical mechanisms for entry into S phase or apoptosis and the regulation of cell cycle duration. A more complete physical description of the cell cycle will enhance understanding of the biochemical pathways that regulate growth.

Most cell-cycle models use multicompartmental analysis. The compartment is analogous to the phase of the cell cycle (i.e. $G_1/S/G_2/M$) and as the cell progresses through the cell cycle, it spends time in each compartment. These compartments can have differing properties depending on the stage of the cell cycle: (i) residence time is fixed or regulated by a rate constant or function; (ii) loss of cells from specific compartments by apoptosis; and (iii) renewal by a binary branching process.

The first models that were developed were based on the fraction of mitoses that were labelled by tritiated thymidine.⁴⁻⁶ Development of a flow cytometric technique that could measure cell division and cell cycle status by bromodeoxyuridine (BrdU) incorporation with quenching of H33258 fluorescence⁷ demonstrated that G_1 shows wide variation in duration in comparison to the rest of the cell cycle, which is relatively fixed (S phase, G_2M). Thus, the duration of G_1 could be modelled as a random variable. This probabilistic interpretation of G_1 transit led to development of stochastic models where entry into S phase was governed by a transition probability.⁸ These stochastic models account for the inherent variability in the duration of the cell cycle.

The model developed here is based on these concepts (Fig. 4). The cell cycle is divided into A and B phases. The time cells spend in the A compartment is described by a rate process. The rate at which cells enter the B compartment (or undergo apoptosis) is directly proportional to the number of cells in the A compartment. The time cells spend in the B

Figure 4 Physical model of cell cycle transit. Each generation is divided into A and B compartments and the number of progenitors in these compartments referred to as a function of time. Cells leave the A compartment by apoptosis or progress to compartment B at a rate determined by the rate constants q and r, respectively. The transit time for compartment B is τ . The number of cells in each generation is found by summing A and B compartments.



compartment is constant. Thus, the rate at which cells leave the B compartment is the same as the rate at which they enter, but delayed by a time lag. Consecutive cell cycles are linked in series.

The method for solving this problem is to formulate a series of first-order differential equations that describe progenitor cell fluxes between the A and B compartments and successive generations. It is convenient to set up these equations using progenitor cell numbers rather than progeny, because it is not necessary to double the number of cells with each division. The rate of change for each compartment is equal to the sum of the progenitor inputs minus the outputs:

$$\frac{d[a_i(t)]}{dt} = r \ a_{i-1}(t-\tau) - (r+q)a_i(t), \ n \ge 2$$
[10]

$$\frac{a_1(t)}{dt} = -(r+q)a_1(t)$$
[11]

$$b_i(t) = r \int_{t-\tau}^t a_i(u) du, \ n \ge 1$$
[12]

where $a_i(t)$ is the number of progenitors in the A compartment of generation *i* at time *t* (*i* = 1, undivided cells), τ , the transit time for the B compartment (time lag), *r*, the rate constant for entry into the B compartment and *q*, the rate constant for apoptosis. These equations state that the rate of change in the number of progenitors in compartment A at $d[a_i(t)]$

time t, dt, is equal to the rate of entry from the prior generation, $r a_{i-1}(t-\tau)$, minus the rate at which they leave by entering either compartment B or apoptosis, $(r+q)a_i(t)$. The integral equation describes the number of progenitors in the B compartment and is calculated by summing the number of progenitors that enter from the A compartment, $r a_i(t)dt$, from time $t - \tau$ to time t.

These equations have a unique solution provided that initial conditions are specified. These are the number of progenitors in compartment A at t = 0, $a_1(0)$, and the prior history of progenitors in the B compartment, $b_1(t)$, for $-\tau \le t \le 0$. These initial conditions were found by assuming that at the start of the tracking experiment, cells at all stages of the cell

cycle were labelled. If it is assumed that cells are cycling asynchronously and the numbers of cells in A and B compartments do not change with respect to time, then the steady state solution will define the initial state.

$$b_1(t) = r\tau a_1(0), -\tau \le t \le 0$$
 [13]

These equations were solved to give an analytical solution for the time evolution of progenitor numbers in consecutive generations.⁹ In the next section, the main findings of this model are summarized.

Relationship between model parameters and measured quantities

The goal of modelling is to understand the physical processes that regulate cell growth and apoptosis. In the present review, we will focus on the physical interpretation of progenitor survival and proliferation that is provided by the model. In this case model parameters r, q and τ are estimated using measured quantities m(t), p(t) and s(t).¹⁰

Following a lag period, one intuitively expects the mean generation number to increase linearly with respect to time, and the proliferation to increase exponentially and survival to decrease exponentially. The model approximates this 'smooth' exponential behaviour provided that 1/(r + q) is not small in comparison with τ .

$$p(t) \approx \exp[k_n(t - \log)]$$
 [14]

$$s(t) \approx 1 - \exp[k_s(t - \log)]$$
 [15]

$$m(t) \approx k_m(t - \log)$$
[16]

where k_p , k_s and k_m are the respective rate constants for proliferation, survival and mean generation number. These rates are estimated using linear regression analysis (Fig. 3). The proliferation and survival per generation are defined:

$$p_1 = \exp\left[\frac{k_p}{k_m}\right]$$
[17]

$$m(t) \approx k_m(t - \log)$$
[16]

These estimated values are closely related to model parameters which are derived from *r*, *q* and τ . The renewal, ρ , which is related to the survival per generation, is defined as the proportion of cells that go from compartment A to B

$$\rho = \frac{r}{r+q}$$
[19]

The stochastic fraction, σ , is related to the proliferation per generation and is defined as the average proportion of the cell cycle that cells spend in the A compartment

$$\sigma = \frac{1}{1 + \tau (r + q)}$$
[20]

The time a cell spends in the A compartment is stochastic, because there is no way of predicting when the cell will leave. It is only possible to estimate the probability of departure given the arrival time. In contrast, the B compartment is deterministic, because it is possible to predict the time a cell leaves from its time of arrival (τ time units later).

Figure 5 shows the relationship between (s_1, p_1) and (ρ, σ) . A family of curves for constant values of either σ (near-horizontal lines) or ρ (near-vertical lines) are used to predict these parameters given the measured quantities s_1 and p_1 . The model has shown that renewal, ρ , is almost equal to survival per generation, s_1 . This ideality is lost if there is significant apoptosis. Proliferation per generation has a range of value going from 2 to *e* (2.71...), depending on whether the cell cycle is predominantly deterministic $(1/(r + q) \rightarrow 0)$ or stochastic $(\tau \rightarrow 0)$. There is also a slight dependence of ρ on p_1 , and σ on s_1 , particularly for high values of p_1 and low values of s_1 .

The model was used to estimate cell cycle parameters for the NALM-6 cell line. Regression lines shown on Fig. 3 were used to calculate the average cell cycle duration and survival and proliferation per generation. The average cell cycle duration of 32.7 ± 0.8 h, (n = 4, 95% confidence interval) was estimated from the reciprocal of the slope of generation



Figure 5 Estimation of model parameters using data. The proliferation and survival per generation are estimated from data (see text) and are plotted using the left y axis and bottom x axis. The model parameters, renewal and stochastic fraction, are estimated using the family of curves that define proliferation and survival per generation for either constant renewal or constant stochastic fraction. These curves are labelled on the upper x axis and right y axis, respectively. Data for the pre-B cell line, NALM-6, has been plotted on this graph.

number versus time (\bigcirc). The values of $s_1 = 0.99 \pm 0.01$ and $p_1 = 2.33 \pm 0.02$ (n = 4, 95% confidence limits) were calculated from the slope of lines using log-transformed survival and proliferation data.

The corresponding model parameters are estimated using the curves (labelled with the top x and right y axes) that lie close to the values of s_1 and p_1 for NALM-6 (Fig. 5). The renewal and stochastic fractions were estimated to lie in the region ($0.97 < \rho < 1$) and ($0.74 < \sigma < 0.79$), respectively.

Conclusions and future directions

Sorting of CFSE-labelled cells using a narrow gate (< 10/256 channels) can increase the resolution of fluorescent division tracking. This procedure will expand the application of this technique to the study of cell types that have heterogeneous staining characteristics. Because only a narrow subset of the entire labelled cell population is tracked, it is important to establish that the sort region does not influence the proliferative behaviour of cells or their phenotype.

This technique provides a powerful method for analysing cell cycle kinetics and divisional recruitment. A time series of cell division tracking histograms can be used for detailed analysis of cell generation time, survival and proliferation. In comparison to DNA analysis, the method provides a more complete picture of cell cycle transit.

Analysis of cell cycle transit by division tracking provides at least three new quantities, which were not easily measured before this technique was established. These are the time between successive divisions, the survival per generation and the proliferation per generation. These quantities can be used to estimate the rates of apoptosis and the fraction of the cell cycle that is stochastic, presumably the result of the G_1/S checkpoint. Future work will determine the adequacy of probabilistic models to predict cell cycle transit times, with possible modifications to include compartments for prolonged survival before apoptosis (i.e. terminal differentiation). This form of analysis will lead to better physical and biochemical models of cell cycle transit.

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