

Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data

Edwin D Hawkins¹⁻³, Mirja Hommel^{1,3}, Marian L Turner^{1,2}, Francis L Battye¹, John F Markham¹ & Philip D Hodgkin¹

¹Immunology Division, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, 3050 Victoria, Australia. ²Department of Medical Biology, University of Melbourne, Melbourne 3010, Victoria, Australia. ³These authors contributed equally to this work. Correspondence should be addressed to P.D.H. (hodgkin@wehi.edu.au).

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Cellular proliferation is an essential feature of the adaptive immune response. The introduction of the division tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE) has made it possible to monitor the number of cell divisions during proliferation and to examine the relationship between proliferation and differentiation. Although qualitative examination of CFSE data may be useful, substantially more information about division and death rates can be extracted from quantitative CFSE time-series experiments. Quantitative methods can reveal in detail how lymphocyte proliferation and survival are regulated and altered by signals such as those received from co-stimulatory molecules, drugs and genetic polymorphisms. In this protocol, we present a detailed method for examining time-series data using graphical and computer-based procedures available to all experimenters.

INTRODUCTION

Much of our understanding of immune regulation has been made from experiments examining lymphocyte proliferation. Traditional *in vitro* methods take a snapshot of the number of dividing cells by pulsing with tritiated thymidine or measuring total cell number indirectly^{1,2}. These methods provide relative information about broad effects on proliferation but give no deep insight into how the proliferative response is being altered. For example, a greater number of cells or greater uptake of tritiated thymidine in culture A compared to culture B could mean any one of the following: (a) more cells are responding in A and the division rate is the same for all cells; (b) cells are entering division earlier in culture A; (c) the division rates are the same in A and B but there is less death in culture A; or (d) the division rate is faster in culture A than B.

These scenarios can be distinguished with the additional information provided by using 5 (and 6)-CFSE division tracking and quantitative flow cytometric methods.

Interpreting CFSE data requires a working model of the underlying dynamic cell changes associated with division. A quantitative model assigns a minimal set of informative parameters to describe the behavior of a proliferating population of cells, thus allowing comparisons of proliferative responses. In recent years, quantitative methods have been applied to many different protocols for stimulating lymphocytes *in vitro*, including both T and B cells of mouse and human origin³⁻⁶. Surprisingly, all lymphocytes tend to follow similar kinetic and stochastic characteristics, which has allowed the development of standardized models that can be used to acquire a great deal of information about lymphocyte proliferation.

Key features of lymphocyte proliferation

The following sections provide a summary of the key features of lymphocyte proliferation and the important model parameters that have emerged from these studies. These parameters form the basis for the experimental and analytical methods described in this protocol.

The time to first division distribution. Inspection of any CFSE profile reveals that both B and T cells typically do not divide

synchronously, but are spread across divisions. The main source of this variation is the time cells take to enter their first division. Thus, the time to first division distribution exhibited by the cell population is an important descriptive feature of any model. Direct measurement of times to divide for purified populations of T or B cells using the colcemid method reveals them to closely approximate a lognormal distribution⁵. In some cases, the more familiar Normal distribution also provides a reasonable fit to the time to first division curve³. Mathematical models that use simple differential equations approximate the variation in entry to first division with a delayed-exponential curve⁷⁻⁹. At present, we do not know how the variation in division times arises from internal or external influences and therefore there is no predictive theory as to the correct or exact distribution. For this reason, the choice of distribution must be made either for empirical accuracy (lognormal distribution) or practical advantage (Normal distribution) or to simplify the mathematical treatment (delayed-exponential). The precursor cohort method that is presented in this article uses a Normal (also known as a Gaussian) distribution to describe time to first division and can be contrasted with the Cyton model¹⁰, which uses the more accurate lognormal distribution.

Progressor fraction. Only a certain proportion of a starting population of cells will have participated in division by the end of an experiment. Some cells do not divide because they die first, others may simply not be stimulated and others may not have reached their 'time to first division' before the end of the experiment. The progressor fraction parameter therefore describes the proportion of cells that participate in division. Models with different levels of sophistication accommodate these alternatives in various ways. In the models described in this article, the precursor cohort method provides an estimate of the fraction of progressing cells that does not distinguish between those that die and those that are unstimulated. Alternatively, the Cyton model provides an estimate of the progressor fraction as if there were no cell death and the simultaneous death rate of undivided cells is described with an additional parameter.

Subsequent division time. Once cells enter the first division, their subsequent divisions usually, but not always, proceed more rapidly and display little variation in the length of cycle times. For strongly stimulated T and B cells, it is often sufficient to assume that the subsequent divisions follow, and can be described by, an exact division time. In contrast, more weakly stimulated cells may exhibit significant variation in subsequent division times.

Cell death. All cell populations placed in culture are subject to some death. This death can be the result of the cell preparation process ('mechanical death'¹⁰), which exhibits itself as a very rapid loss of a proportion of undivided cells. Alternatively, death of undivided cells may be more protracted and due to deprivation of survival factors found *in vivo*^{3,11}. The simplest distribution describing such loss is the exponential and follows a curve akin to that of radioactive decay³. This approach is used in the precursor cohort method and allows the survival of cells under different conditions to be expressed as a half-life. More accuracy can be achieved by assuming time-dependent survival distributions¹⁰.

Death of dividing cells. The survival of dividing cells also varies under different conditions. The precise nature of how survival and division interleave remains unknown. The precursor cohort method provides a method of estimating the proportion of cells lost per division. The Cyton model assumes that a population of dividing cells has an intrinsic time-to-die parameter that can be described by a probability distribution that takes the start of each subsequent division as time zero.

Additional considerations

The experimenter needs also to be aware that there are possible points of departure from the typical lymphocyte proliferation behavior described above. Some examples of nonstandard behavior include the following:

(1) *Limited proliferation.* Proliferation of primary lymphocytes cannot be sustained indefinitely in culture. Proliferation may be restricted owing to limitations of nutrients in the culture medium or overcrowding of the culture vessel. There are also situations in which the number of divisions appears to be regulated by the strength of initial stimulation or the continued presence of stimulation¹⁰.

(2) *Change of conditions with time.* If proliferating cells produce a growth factor capable of influencing their own growth, then deviations from the expected outcomes will likely occur.

(3) *Cell density.* Contact between cells may affect proliferation parameters and care must be taken to develop culture conditions that minimize such effects if comparisons are to be made.

(4) *Division-linked changes.* Early division-tracking experiments with CFSE revealed that cell differentiation can be linked to the progression of cells through successive divisions^{12,13}. An emerging differentiated population may have different proliferation and/or survival properties to undifferentiated cells, resulting in nonstandard outcomes. Similarly, any kinetic parameter may potentially be linked to division in this way, even without differentiation. Thus, division times, or times to die, could progressively change with division in some situations.

When examining the underlying kinetic behavior of a cell population in different stimulatory conditions, it is preferable to work in a minimal system to ensure that all variables can be adequately controlled. With this in mind, much of the quantitative

work performed to date has been conducted *in vitro*. In principle, the same methodology can be applied to data from *in vivo* experiments. However, the greater complexity of an *in vivo* system (e.g., the inability to distinguish between cell death and cell migration) will likely make the interpretation of the results of these experiments less definite.

It is not possible to anticipate all of the alternative potential outcomes from a quantitative analysis of a CFSE data series. However, the methods provided here are a valuable starting point for investigating and comparing lymphocyte proliferation in various stimulation conditions. The following protocol describes how to conduct thorough CFSE time-series experiments, analyze CFSE profiles to extract cell numbers and study the resulting data to extract rates of division and death. We favor the use of both graphical methods and automated model fitting. The former method facilitates a familiarity with the data and reveals trends and differences in a visual, intuitive way. When data are known to conform to a particular model, then direct computer-aided fitting and parameter estimation are both time-efficient and accurate.

Considerations before obtaining data

Experimental design is important. Triplicate cultures for each culture condition at each time point increase the accuracy of the assay. The following factors should be considered when designing the experiment.

Time points: A typical CFSE time-series experiment requires 8–12 time points spaced 6–12 h apart. Choose harvest times based on the required information from the experiment. The first time point should be taken at approximately 5 h, as this gives a more accurate starting cell number than a count at 0 h owing to the immediate initial 'mechanical' death that can sometimes be observed¹⁰. For examination of early survival and proliferation behavior, it is necessary to have multiple time points up to and including the first division (~40 h). For analysis of the extent and duration of the entire response, it is ideal to have two time points per day until the experiment is complete.

Starting cell number: A minimal starting cell number must be chosen that is appropriate for the stimulation conditions. The ideal starting cell number will vary between cell type and stimulation condition. This must be low enough to allow multiple rounds of cell division before overgrowth but not so low as to jeopardize interactions that are cell density-dependent and required for proliferation.

Controls: Appropriate analytical controls should be chosen. This is described in detail in the accompanying article¹⁴.

Overview of the procedure

Having acquired the data for each harvest time point (Steps 1 and 2), there are two stages in the further analysis. The first stage (Steps 3–7) is to analyze each harvest time point individually, calculate the total number of live and dead cells for each stimulation condition and finally to calculate the number of cells in each division on the basis of CFSE profiles. In the second stage (Step 8), the data from each time point can be compiled for analysis of the full time course. This enables information about other proliferation parameters, including division and death rates, to be obtained. Key to this analysis is adopting a model of the underlying growth and survival dynamics. We describe two methods, the first method (Steps 8A and B) provides information intuitively using

a graphical method called the precursor cohort method. The second method (Step 8C) involves the use of an existing software package that allows data to be fitted directly using a model of lymphocyte proliferation: this is called the Cyton Calculator method.

Precursor cohort method: In this article, we present two options for analyzing precursor cohort plots (Steps 8A and B). Option A describes a method suitable for the basic user, which utilizes in-built statistical tools in software such as GraphPad Prism (Graphpad Software Inc.) and Microsoft Excel (Microsoft). Option B describes an advanced method that users with sophisticated mathematical skills will be able to implement in suitable platforms such as C++, R and Matlab (Mathworks). This advanced method allows more thorough statistical analyses such as calculation of 95% confidence intervals. The precursor cohort method utilizes plots that illustrate the progression of the unsynchronized population of cells across divisions based on their CFSE profiles. CFSE profiles are normalized by dividing the cell numbers by $2^{\text{division number}}$ to remove the effect of cell expansion (Step 8A(i)). Therefore, the behavior of the starting population of cells can be tracked through divisions. These plots are called precursor cohort plots. This method assumes a model of cell proliferation whereby cells follow a Normal distribution of time to enter first division and divide regularly at each subsequent division. Survival parameters are expressed as a half-life before first division and as a deterministic proportion of cells dying through each subsequent division.

The following basic parameters can be estimated using this method: (a) time to first division (expressed as a Normal distribution); (b) the subsequent division time of cells that enter division; (c) the half-life of cells that die before first division; (d) the rate of cell death as they are undergoing subsequent divisions; and (e) the proportion of starting cells that will proliferate.

Cyton Calculator method: A model of proliferation and survival can be fitted directly to the data set obtained at the end of Step 6, and optimal values for parameters describing proliferation and survival can be determined using standard nonlinear fitting techniques such as minimizing sums of squares. The Cyton model developed by Hawkins *et al.*¹⁰ provides a more accurate description of proliferation and survival parameters than those used for the precursor cohort method, although, similar to all models, it makes assumptions regarding the underlying operation of the cell. The Cyton model uses lognormal distributions to describe time to divide and die in the first division and for each subsequent division. The ability to fit this model to experimental data sets is offered in ‘The Cyton Calculator’ software available for free download from our website. The model also introduces a division destiny parameter, a distribution that describes the maximum number of divisions a cell population undergoes, and therefore it can describe data sets in which cells reach a natural limit to proliferation¹⁰. As this is a complex and nonlinear model with many parameters, time should be taken to become familiarized with the model and the program.

MATERIALS

REAGENTS

- Purified B or T cells labeled with CFSE and treated as required for your specific experiment in one 96-well tissue culture plate per time point
- Medium and medium supplements for cell culture (see accompanying article¹⁴)
- Propidium iodide (PI; Sigma)
- Calibrite beads (Becton Dickinson)

EQUIPMENT

- 5 ml polystyrene round-bottomed tubes for FACS analysis (Becton Dickinson)

- Flow cytometer
- Multichannel pipette
- FACS analysis software—FlowJo (Tree Star) or WEASEL (WEHI)
- Data-fitting software such as GraphPad Prism (Graphpad Software Inc.) or other suitable statistical packages
- Spreadsheet software such as Microsoft Excel (Microsoft)
- Cyton Calculator (for Step 8C only; WEHI; http://www.wehi.edu.au/WEHI_Groups/workgrp/cyton/website/pages/cc.html)

PROCEDURE

Harvesting of time points

1| Add Calibrite beads to wells immediately before cell harvest and flow cytometric analysis to allow determination of cell number. Correct addition of beads to culture is crucial for accurate time-series data. The number of beads added to the culture should be in the range of 5–10% minimum of the total events measured in the flow cytometry files and should be added to the culture in 10–50 μl of PBS. Prepare a stock of beads that is sufficient for use throughout the entire experiment. Mix the bead stock thoroughly by pipetting before adding to each well. Within one time-point harvest, beads should be added to all wells with the same pipette tip to reduce error. Prepare a stock of PI at 1 $\mu\text{g ml}^{-1}$ in PBS and add 10 μl to each well. Mix the cells and beads in each well thoroughly using a pipette (avoiding bubbles) and transfer samples to individual 5 ml polystyrene round-bottomed tubes. It is not essential to harvest the entire sample, as cell numbers will be determined using a ratio of cells to beads.

Collection of data

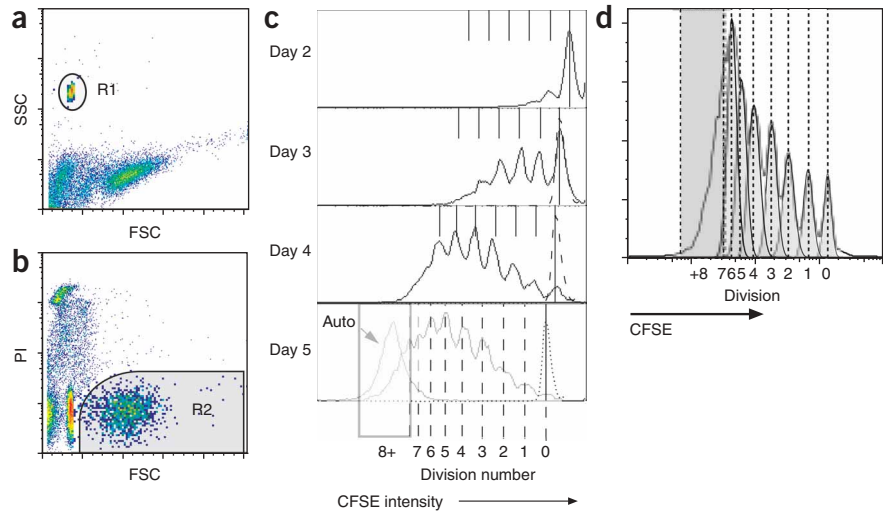
2| Set up the flow cytometer with instrument settings suitable for distinguishing the populations outlined in **Figure 1**. First set up a forward scatter (FSC)/side scatter (SSC) dot plot to allow a clear distinction of live and dead cells as well as beads. This dot plot should be used to set a gate for beads (**Fig. 1a**). An FSC/PI (FL-3) dot plot should be set up to set gates on live and dead cells (**Fig. 1b**). A region should be set using the live cell gate and then used for an FL-1 histogram to show CFSE peaks (**Fig. 1c,d**). These settings should be saved and used for all time points in an experiment.

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Figure 1 | Analysis of FACS data. CFSE-labeled B cells were stimulated with α CD40 + IL-4 and harvested in the presence of beads/PI as described. **(a)** FSC/SSC plot with gate R1 indicating the position of the beads. **(b)** The same data illustrated in **a** are presented as an FSC/PI plot to exclude dead cells. Gate R2 includes all live lymphocytes. **(c)** CFSE time-course data of stimulated T cells. Note the slow decay of CFSE intensity as demonstrated by the decline in the fluorescence of the undivided peak (solid line). The location of autofluorescence of stimulated unlabeled cells on day 5 illustrates the difficulty in resolving divisions greater than 7. Thus, we recommend an 8+ division gate (shaded box). **(d)** CFSE profile from R2 gate illustrated fitted Gaussians to determine gates for division number.



3 | Pipette each sample immediately before running to allow mixing of cells and beads. Collect 10,000–20,000 events per sample. The acquired data file should contain all information collected, not just data from specific gates.

Calculating cell numbers per division at each time

4 | Open the acquired FACS data in FACS analysis software. Set the gates on beads and live cells (**Fig. 1a,b**). An FL-1 histogram on live cells will show CFSE peaks (**Fig. 1c,d**). Gates need to be drawn for each CFSE peak; this can be done either manually or by using a peak fitting function if available in the analysis software (see **Fig. 1d** and the accompanying article¹⁴). Prepare a table for the number of live cells in each division class (see **Table 1** for a typical example).

▲ CRITICAL STEP If using a fitting function, care should be taken to manually set the gate for division 0, a value for CFSE autofluorescence (this can be obtained using non-CFSE-labeled cells), and to fit the number of division gates (see **ANTICIPATED RESULTS**). Typically, the overlap near the autofluorescent level makes it difficult to resolve divisions higher than about 7. The cells falling outside the final position should be recorded as greater than the last division counted (**Fig. 1c,d**).

▲ CRITICAL STEP Gates need to be checked for each replicate FACS file within a time point. Gates should also be checked between time points; it is common that the division 0 peak will shift to a slightly lower fluorescence over the duration of a time course as discussed in the accompanying article¹⁴.

5 | Calculate actual live cell numbers using a ratio based on the number of beads added to the cultures before harvest: total number of cells in culture well (within live or dead gate) = beads added/beads counted (within bead gate) \times cells counted (within live or dead gate).

▲ CRITICAL STEP For accurate determination of cell numbers, the number of beads added must be known and must be reproducible over the course of the experiment.

TABLE 1 | Typical data from CFSE time-series experiments.

Division	Harvest time (h)							
	0	24	32	48	56	66	75	85
0	50,000	14,807	9,160	1,777	429	35	2	0
1	0	527	1,861	3,920	2,351	541	65	3
2	0	75	560	5,191	6,501	3,762	1,045	104
3	0	3	66	2,731	7,161	10,401	6,627	1,672
4	0	0	1	566	3,129	11,457	16,710	10,603
5	0	0	0	44	537	5,006	16,787	26,736
6	0	0	0	0	33	859	6,684	26,859
7	0	0	0	0	0	52	1,044	10,695
8	0	0	0	0	0	0	54	1,671
Total	50,000	15,412	11,648	14,229	20,141	32,114	49,018	78,342



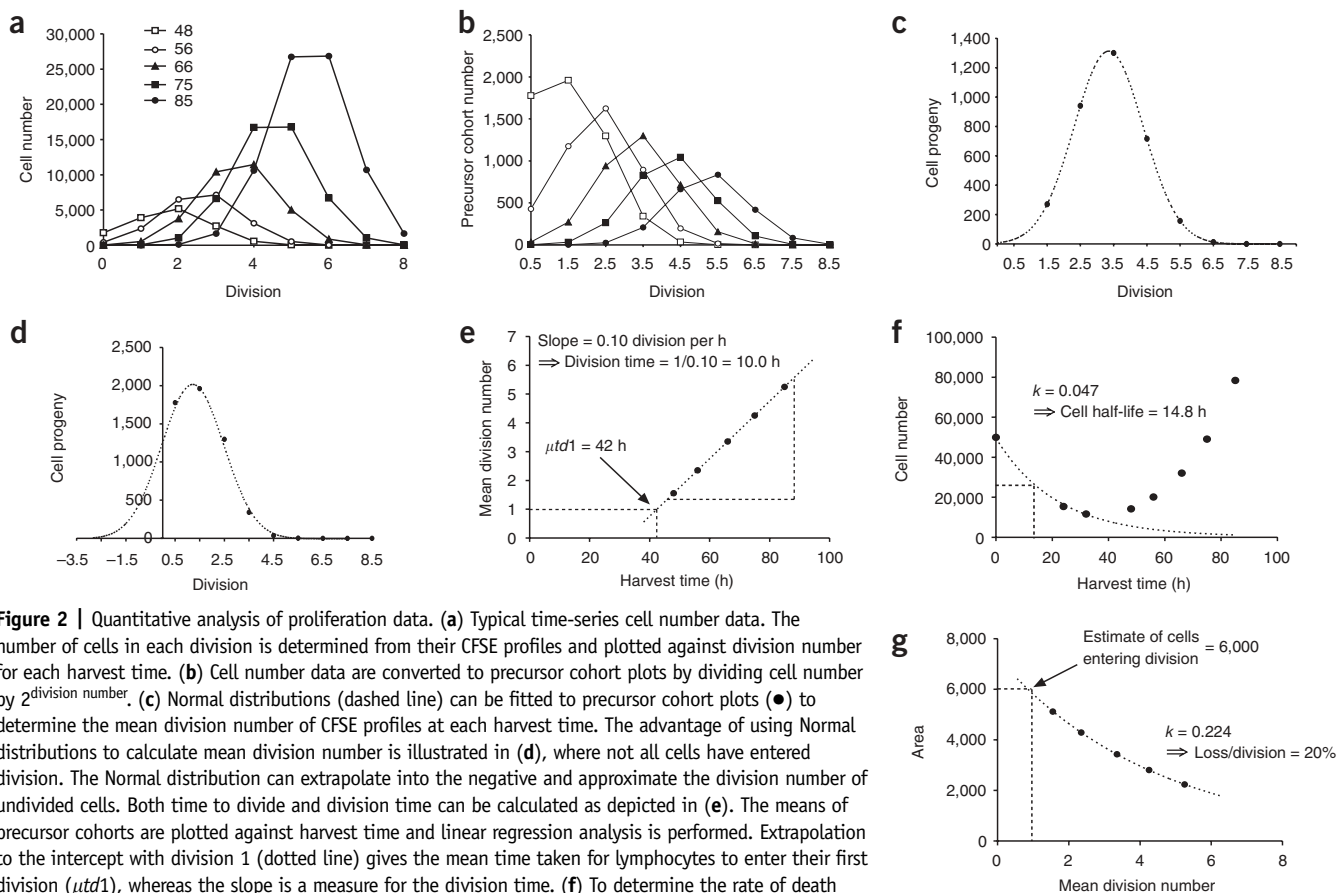


Figure 2 | Quantitative analysis of proliferation data. **(a)** Typical time-series cell number data. The number of cells in each division is determined from their CFSE profiles and plotted against division number for each harvest time. **(b)** Cell number data are converted to precursor cohort plots by dividing cell number by $2^{\text{division number}}$. **(c)** Normal distributions (dashed line) can be fitted to precursor cohort plots (●) to determine the mean division number of CFSE profiles at each harvest time. The advantage of using Normal distributions to calculate mean division number is illustrated in **(d)**, where not all cells have entered division. The Normal distribution can extrapolate into the negative and approximate the division number of undivided cells. Both time to divide and division time can be calculated as depicted in **(e)**. The means of precursor cohorts are plotted against harvest time and linear regression analysis is performed. Extrapolation to the intercept with division 1 (dotted line) gives the mean time taken for lymphocytes to enter their first division ($\mu td1$), whereas the slope is a measure for the division time. **(f)** To determine the rate of death before division entry, total cell numbers are plotted against harvest time and an exponential fitted. **(g)** Both the approximate number of cells entering division and the loss of cells per division can be estimated by plotting the areas of the precursor cohorts against the mean division numbers.

6| Calculate the actual cell numbers in each CFSE division peak: total number of cells (within division peak) = total number of cells (from Step 5) × fraction of total cells in division (from Step 4).

7| Repeat the calculations (Steps 5 and 6) for each culture condition at each time point. An example of tabulated data representing the means of triplicates is shown in **Table 1**. A plot of typical data is shown in **Figure 2a**. If using Microsoft Excel, to save time, spreadsheets can be devised to allow automated calculations of cell numbers as well as means and standard deviations from the means of triplicate cultures.

Analysis of data for effects on proliferation/survival

8| Once a table of cell numbers per harvest time is created, if desired, subject the data to further analysis to recover information about other proliferation parameters, including division and death rates. Two methods are offered here: options A and B use the precursor cohort method, whereas option C uses the Cyton Calculator method. If using a precursor cohort method, option A is most suitable for the basic user, whereas option B allows more thorough statistical calculation of mean division numbers of precursor cohort plots.

(A) The precursor cohort method: basic method

(i) Generate a table of precursor cohort numbers by dividing the total number of cells in the i th division by 2^i , where i is the division number. Add 0.5 to division numbers (see **CRITICAL STEP**). An example of these data is shown in **Table 2** and **Figure 2b**.

▲ CRITICAL STEP CFSE analysis does not distinguish between cells that have just divided and those late in their division cycle. However, for quantitative analysis, a continuous scale of division progression must be introduced. For example, a cell that has just entered division 3 will be in division 3.0, halfway through its division it will be in division 3.5 and just before dividing it will be in division 3.95. Note that all the above stages are recorded simply as division 3 when referenced to CFSE profiles. Calculations of the mean division number need to take account of this continuous scale, so a mean such

TABLE 2 | Time-series data converted to precursor cohort format.

Division	Harvest time (h)							
	0	24	32	48	56	66	75	85
0.5	50,000	14,807	9,160	1,777	429	35	2	0
1.5	0	263	930	1,960	1,176	270	33	1
2.5	0	19	140	1,298	1,625	941	261	26
3.5	0	0	8	341	895	1,300	828	209
4.5	0	0	0	35	196	716	1,044	663
5.5	0	0	0	1	17	156	525	836
6.5	0	0	0	0	1	13	104	420
7.5	0	0	0	0	0	0	8	84
8.5	0	0	0	0	0	0	0	7
Total	50,000	15,089	10,239	5,413	4,338	3,433	2,806	2,244

as 2.3 divisions is possible. The half division scale is applied, as on average cells in a division are halfway between their division cycle.

- (ii) Fit Normal distributions to the precursor cohort plots (**Fig. 2c**) for each time point. The distribution should be fitted only to data for cells in division (i.e., leave out division 0 data). Allow the fitting software to extrapolate the distribution to negative division values when necessary (**Fig. 2d**).

▲ CRITICAL STEP Estimating the average division number of the dividing cohorts of cells is complicated by the fact that many of the precursor cohort plots will have only a partial or truncated Normal distribution. The mean of these distributions must therefore be estimated as though they were complete, which potentially involves extrapolating to negative values, as discussed previously and illustrated in **Figure 2d**.

■ PAUSE POINT Fitting Normal distributions can be achieved using a data-fitting software package such as GraphPad Prism (Graphpad Software Inc.) or other suitable statistical analysis packages. A basic understanding of the fitting software is required such that the means, standard deviations and areas of the Normal distributions can be extracted. The remaining plots and trendlines can be drawn and fitted in a spreadsheet such as Microsoft Excel. A more advanced procedure for calculating the parameters required for this method directly from data from Step (i) is given in Step 8B.

- (iii) Tabulate results as shown in **Table 3**.
- (iv) Plot mean division number versus harvest time (**Fig. 2e**). Only use harvest times that have sufficient data to fit a Normal distribution, that is, plots with a reasonable number of cells participating in division (times > 48 h in the example; **Fig. 2e**). A general rule to work by is that mean division numbers > 1.5 will give the most accurate results.
- (v) If the plot from Step (iv) is linear, fit a trendline and calculate the slope and intercept with division 1. Possible reasons why a linear plot is not produced are discussed in the 'Practical applications of the precursor cohort method' section. Trendline fitting can be done automatically in Microsoft Excel by right clicking on the line and selecting 'add trendline'. The equation for this fitted line can be displayed by selecting 'display equation' in the options menu and will typically be in the standard equation for a line format $y = mx + c$. The apparent time between subsequent divisions is given by $1/m$ (**Fig. 2e**). The apparent mean time to first division ($\mu td1$) is given by the intercept of this plot with division 1. This can be calculated by solving the equation $\mu td1 = (1 - c)/m$ (**Fig. 2e**).
- (vi) Estimate the half-life of cells leading up to first division. Choose harvest times before division begins (< 30 h in the example). Plot total live cell number against time of harvest (**Fig. 2f**). Fit an exponential function (as described previously). This equation will typically be in the format $y = e^{-kx}$, where k is the exponential decay constant. Calculate the half-life of cells that die before first division using the following formula: half-life = $(\ln(2))/k$.
- (vii) Estimate the rate of cell loss per division. Plot the area of the Normal distributions fitted to the precursor cohort plots (where area is represented as cell number) versus the corresponding mean division number. If a constant proportion is lost at each division, an exponential pattern will be obtained (see **Fig. 2g**). Fit an exponential trendline to these data (as described previously). The proportion of cells dying per division can be estimated using the following formula: death per division = $1 - e^{-k}$.
- (viii) Estimate the proportion of starting cells that will enter first division. The plot (**Fig. 2g**) from Step (vii) can be

TABLE 3 | Typical data set after precursor cohort analysis.

Harvest time	Mean division number	SD	Area
32	-0.61	1.060	15,958
48	1.55	1.041	5,120
56	2.35	1.042	4,290
66	3.35	1.042	3,432
75	4.25	1.042	2,807
85	5.25	1.060	2,235



extrapolated to intercept with division 1. This number is an estimate of the expected number of cells entering first division.

▲ CRITICAL STEP A Microsoft Excel worksheet ‘STEP-BY-STEP’ and the set of data presented in this article can be downloaded from our website (http://www.wehi.edu.au/WEHI_Groups/workgrp/cyton/website/pages/pcm.html), which allows easy calculation of the parameters of the precursor cohort method directly from data obtained from Step 7.

(B) Calculating mean division number from cohort data: advanced method

- (i) Fit the cohort division numbers to a probability weight function P_i , constructed from the Gaussian probability density function, where μ is the mean and σ is the standard deviation, as follows:

$$P(n) = \left(\frac{\exp(-(n - \mu)^2 / 2\sigma^2)}{\sigma\sqrt{2\pi}} \right)$$

- (ii) Perform a least squares fit, whereby the function

$$\chi^2(\mu, \sigma, \alpha) = \sum_{i=1}^N (w_i - \alpha P_i)^2$$

is minimized, ignoring the undivided cells ($i = 0$). Here α is a weighting representing the fraction of the cohort that divides, where w_i are data points and

$$P_i = \int_{n_i}^{n_{i+1}} P(n) dn$$

- (iii) This least squares fit yields parameter values for μ , σ and α .
- (iv) If an estimate of confidence in the values of the parameters obtained from the above fitting is required, a Monte-Carlo bootstrap method can be used. This involves repeating the fit a number of times, each time with a set of synthetic data chosen randomly from a Normal distribution with a mean given by the measured data point, and a standard deviation given by the standard deviation of the residuals from the original fit. In this way, we obtain a distribution of possible values for each parameter, from which we can obtain the mean and standard deviation for each of the estimated parameters. The standard deviations obtained in this way can then be used as weighting for fitting in the subsequent linear regression analysis (Steps 8A(iv) and (v)).

▲ CRITICAL STEP If the average number of divisions is less than 2, the ability to find a reasonable Normal distribution is limited. For this reason, the analysis and conclusions should focus on data sets where the average division number is greater than 2. It is also possible to take the standard deviation from later time points and fix that value when fitting to earlier time points. If this method is to be used, care needs to be taken with the possibility for overestimating the standard deviations.

- (v) Use linear regression techniques to fit a line to the data for the mean division number as a function of time, as mentioned in Step 8A(v), using the equation

$$y = mx + c$$

This is done with a weighted sum of squares using the mean division number μ_i and the standard deviation in this value s_i , obtained from the Monte-Carlo bootstrapping method, by minimizing the function

$$\chi^2(m, c) = \sum_{i=1}^N \left(\frac{y_i - mx_i - c}{s_i} \right)^2$$

where n_i is the calculated average division number and s_i is the standard deviation in that value, as calculated above.

- (vi) From the parameters obtained from the time to first division, calculate $utd_1 = (1 - c)/m$ and the division time $1/m$. Confidence limits on these values can be calculated using the standard deviations for the mean division number s_i , assuming that there is no error in the time measurements.
- (vii) If you wish, use the mean division numbers from this more advanced method to calculate cell loss through divisions and apparent proportion of responding cells (Steps 8A(vii) and (viii)) using the graphical techniques described previously.

(C) Fitting data directly to a model using Cyton Calculator

- (i) Open the Cyton Calculator. Open the data set to be analyzed. Select ‘display cell proliferation and death graphs’ and view ‘commonly viewed graphs’. This will allow the user to visualize the real data and ‘Cyton Calculator’ model estimates.
- (ii) Adjust various parameter values using the slide controls or directly input plausible parameter values. Use the ‘model fit’ button to optimize parameter values to obtain the best possible fit.

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- (iii) First attempt to fit the data with a minimal set of parameters. For example, some data sets may be able to be fit without a value for division destiny. Restricting the variation in time to subsequent division and cell death in subsequent divisions may also help fitting of parameters describing entry to division. The parameters describing subsequent division behavior can then be added back as needed or desired.
- (iv) Use the 'model fit' button to obtain parameter values that give the best fits possible and record the sum of squares.

TIMING

Assay setup: 1–2 h

Harvesting and FACS analysis at each time point: 1 h, depending on the number of conditions

Data analysis to obtain cell numbers: 3 h

Analysis of cell number data by precursor cohort analysis or Cyton modeling: a typical data set can take 3–5 h to analyze; however, the results of precursor cohort analysis are often revisited and hence the total time required may increase

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	CFSE fluorescence is off the scale	At very early time points, cells will have very bright CFSE fluorescence By 12–15 h, the CFSE fluorescence will be in a useful range	Do not adjust FL-1 voltage at early time points Use FSC/SSC and FSC/PI dot plots to set gates for beads and cells at early time points
2	CFSE autofluorescence is reached after very few rounds of cell division	For adequate distinction of multiple CFSE peaks, the starting fluorescence needs to be $\geq 10^3$ Labeling very small numbers of cells with CFSE sometimes leads to poor starting fluorescence	Increase the number of cells being labeled Follow instructions on CFSE labeling (temperature, duration, concentration) precisely ^{14,16}
2	There are many live cells in the autofluorescence region	Once cells have divided a certain number of times, division peaks are no longer distinguishable	See ANTICIPATED RESULTS
8C(ii)	The fitting tool in Cyton Calculator gives inappropriate parameter values or a very poor fit	The Cyton Calculator has a fitting tool that will function reasonably only if approximately reasonable parameter values are given as a starting point	Estimate parameter values for time to first division, subsequent division time and division destiny on the basis of experimental observations or precursor cohort method parameters

ANTICIPATED RESULTS

The precursor cohort method

Interpreting the mean division versus harvest time plot (Step 8A(iv)). The result of this plot is usually linear; however, if cultured for long periods, the proliferation of lymphocytes will slow down owing to depletion of culture nutrients. This will be apparent as a flattening of the plot (shown in **Fig. 3a**). Some lymphocyte cultures also display a limit to the number of divisions reached that is not due to culture depletion and will also show a plateau. Additional experiments are required to determine the cause of a plateau in division progression. Only the range of harvest times falling in the linear part of the curve should be used to calculate the division times and the apparent time to first division.

In the linear portion of this plot, the slope of the line of best fit is equal to the reciprocal of the division time (**Fig. 2e**), that is, a shallower slope represents a slowly dividing population, whereas a steeper gradient indicates a faster dividing population. The intersection of the line of best fit with division 1 on the y axis gives an estimate of the mean time to first division (**Fig. 2e**). In the illustrated example in **Figure 2e**, the equation for the fitted trendline is $y = 0.1x + (-3.24)$ (using the standard $y = mx + c$ nomenclature). Therefore, the division time = $1/0.1$ or 10 h, whereas $\mu td1 = (1 - (-3.24))/0.1$ or 42 h. However, it should be noted that both parameters can be affected by significant death of dividing cells, and for this reason we say the calculation is of an 'apparent' value (discussed later).

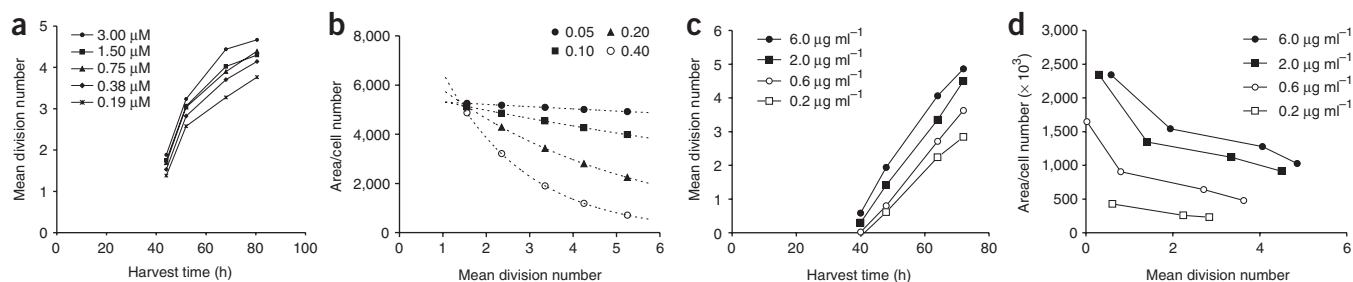


Figure 3 | Experimental situations. **(a)** B cells stimulated with various concentrations of CpG. This experiment represents a common occurrence in *in vitro* cultures. Cells cease proliferating owing to extrinsic factors such as exhausted access to stimuli or space or internal regulation of division number. Hence, the progression of cells through division halts, and a plateau is observed in mean division number. **(b)** An increase in cell loss per division will lead to a steeper fitted exponential curve. **(c,d)** CFSE-labeled OT-I CD8 T cells were stimulated with various concentrations of peptide in the presence of 100 U ml⁻¹ IL-2 and analyzed using the described cohort methods. Graphical analysis illustrates that reducing the peptide concentration delays the time taken to enter the first division (as determined by a delay in the intercept with 1 on the y axis) and the speed of subsequent division times (as illustrated by a reduction in the gradient of the fitted line). Also depending on the culture conditions, a substantial reduction in the areas of precursor plots may be observed, indicating that a proportion of cells are lost during division (as in **b**). In other cases, only relatively few cells enter division as a result of a low progressor fraction or a late time to first division. In these situations, cell numbers become difficult to analyze (**d**).

Interpreting the loss of cells plot (Step 8A(vi)). Unstimulated lymphocytes tend to die in culture at an approximately exponential rate. Even stimulated cells will often follow this rate of death before division, significantly reducing the proportion of the starting cells that participate in proliferation. Under some conditions, the rate of cell loss is altered. For example, in B-cell cultures, the presence of cytokines such as interleukin-4 (IL)-4 or stimuli such as lipopolysaccharide (LPS) can significantly reduce the rate of loss. The plot generated in Step 2 helps determine whether measured differences in the frequency of proliferating cells between two different culture conditions are due to effects on cell survival or sensitivity to the stimulus. The use of an exponential survival curve is convenient, as it provides a reasonable fit to the data. However, for greater accuracy, other skewed probability distributions can be used, such as the lognormal (see ref. 10 for further discussion). Once the exponential curve is fitted, the decay constant k can be used to calculate the half-life of the cells. For example, in **Figure 2f**, the exponential constant $k = 0.047$. Therefore the half-life of undivided cells is $\ln 2/k$ or 14.8 h.

Interpreting the plot of area/cell number versus mean division number (Step 8A(vii)). Plotting the mean division number against the corresponding area of fitted Normal distributions can be used to quantify cell loss through death (**Figs. 2g,3b**). As the cohort method follows the fate of the original starting cells, a reduction in the area of fitted Normal distributions as the time course progresses indicates that cell death is occurring. In contrast, a constant area suggests that there is little cell death occurring as cells divide. A range of possible outcomes is shown in **Figure 3b**. If the loss of cells through subsequent divisions is a constant proportion per division, the expected outcome of this plot is an exponential distribution (**Figs. 2f,3b**). Cell death can be regulated in culture and may be affected by division number and/or culture conditions. In some situations, the pattern of cell loss may not follow an exponential but may appear to change and increase with division number (which could also be affected by time). Further experiments will be required to characterize the reason for the pattern. In the example shown in **Figure 2g**, the decay constant of the fitted trendline is $k = 0.224$. Therefore, the death per division can be calculated using the following formula: death per division = $1 - e^{-0.224}$ or 0.20 or 20%.

In Step 8A(viii), it is suggested that this plot can be extrapolated to division 1 to indicate the number of the starting cells that will enter first division. This can be done easily if there is little evidence of cell death and if the plot is flat, or if it follows a clear pattern such as the exponential. Even then, the significant 'gray area' in obtaining good estimates from cohort plots with mean values of two divisions or less means that this value for cell number entering first division is unlikely to be accurate and is offered for calculation as a guide only.

Practical applications of the precursor cohort method

The precursor cohort method of analyzing data from CFSE experiments is particularly well suited to making direct comparisons between the effects of various stimulation conditions on proliferation parameters. Examples of a cohort analysis performed on data obtained from OT-1 T cells stimulated with different concentrations of peptide and 100 U ml⁻¹ IL-2 are shown in **Figure 3c** and **d**. **Figure 3c** demonstrates that reducing the concentration of peptide in T-cell cultures reduces the speed of subsequent division speed (ranging from 7.5 h per division at 6.0 μg ml⁻¹ to 10.6 h at 0.2 μg ml⁻¹). The mean time to first division is also delayed significantly when the concentration of peptide is reduced (42.1–51.8 h). This set of data example also illustrates the possible outcomes when death is analyzed using graphical methods (**Fig. 3d**). As the concentration of peptide is reduced, the proportion of cells lost through subsequent divisions steadily increases from 16% at 6.0 μg ml⁻¹ to 30% at 0.2 μg ml⁻¹.

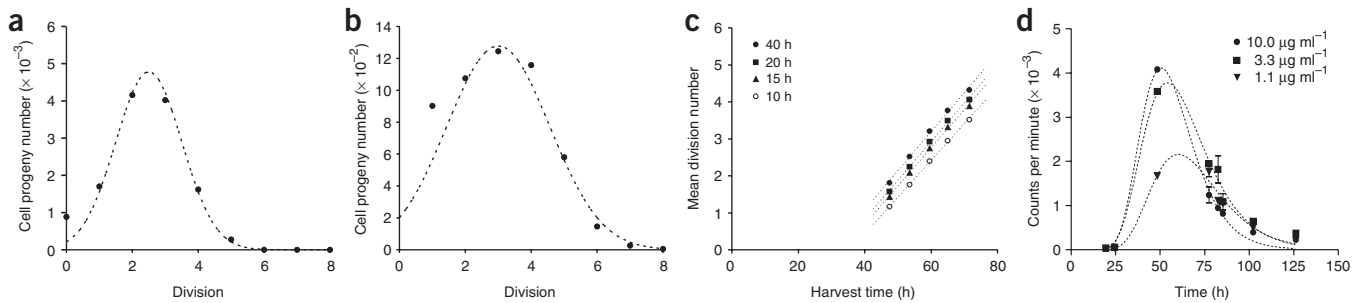


Figure 4 | Caveats to quantitative analysis. Normal distributions (dashed lines) were fitted to cohort data from (a) CD4 T-cell cultures stimulated with $10 \mu\text{g ml}^{-1}$ αCD3 and 100 U ml^{-1} IL-2 and (b) B-cell cultures stimulated with $15 \mu\text{g ml}^{-1}$ LPS. Note the greater variation observed in LPS-stimulated B-cell cultures. (c) Mock data generated with the Cyton model¹⁰ were analyzed using the precursor cohort method. The means of subsequent death times were altered as shown, whereas all other parameters were kept constant. Note the delay in μtd1 in cultures where dividing cells die earlier in subsequent divisions. (d) The μtd1 of B cells stimulated with various concentrations of αCD40 has been directly measured using the colcemid method described in Deenick *et al.*⁵. Conducting this assay in conjunction with CFSE time courses enhances the strength of conclusions that can be drawn from the precursor cohort method.

The intercept of fitted lines with 0 also indicates that increasing the concentration of the peptide increases the number of cells entering the first division.

These methods provide excellent readouts for comparison of division times and death rates within time-course experiments; however, there are limitations that should be considered. This technique is built on the assumption that the variation in time to first division can be approximated using a Normal distribution. In T-cell assays, although the entry to first division is better described by a lognormal distribution⁵, the data can be reasonably well approximated using a Normal distribution, as the variation observed is relatively low. However, B-cell cultures and T-cell cultures with low levels of stimulation exhibit a greater variance in time to first division and a significant departure from the Normal distribution^{3,5}. Therefore, precursor cohort plots from these cultures are subject to errors when fitted by a Normal distribution as illustrated in **Figure 4a,b**. Nevertheless, Normal distributions can still be used to approximate the mean division number and area although with slightly more error.

Mutually exclusive factors controlling lymphocyte proliferation can affect the readouts using these methods. For example, the mean time to first division can appear to be delayed in cultures with large death rates in dividing cells, despite there being no actual change in the time to first division. This is demonstrated in **Figure 4c** for theoretical data created using the model described in ref. 10. Similarly, if a significantly large proportion of cells fail to progress through division, the accuracy of the method is reduced. Thus, data from cultures with low levels of stimulation may be difficult to analyze. The analysis of cohort data in conjunction with assays measuring the time to first division (e.g., the colcemid method described in Deenick *et al.*⁵) therefore aids in constraining parameters of lymphocyte proliferation and significantly increases the accuracy of analysis. An example of data obtained from the colcemid method is shown in **Figure 4d**, demonstrating the usefulness of this technique for identifying changes in mean time to first division and variation.

Estimates of error bars from precursor cohort data

When applying the bootstrapping method to cell number data, a range of fits is generated that provide confidence intervals for the estimated parameters: mean division number, standard deviation and area/cell number. When expressed as 95% confidence intervals, these bounds can be introduced into the weighted linear fit for the plot of mean division number versus time of harvest (Step 8A(iv) and **Fig. 3c,d**). The advantage of undertaking a more rigorous statistical approach is that hypothesis testing methods can be used to establish the likelihood of two values being different.

Fitting data directly to a model using Cyton Calculator

The cohort method provides a simple step-by-step approach for the quantitative analysis of proliferation data. More sophisticated analysis and hypothesis testing require a parametric model of the underlying dynamic process in question. To this end, our laboratory has spent considerable time dissecting cell proliferation data based on CFSE experiments to develop suitable general models that are applicable for most applications. We have also developed software with user-friendly interfaces that can apply appropriate models to data tabulated in the form generated in Step 8. The strength of the Cyton Calculator tools lies in its ability to make quantitative models accessible to the everyday researcher. The Cyton Calculator, instruction booklet and sets of test data are freely available for download at http://www.wehi.edu.au/WEHI_Groups/workgrp/cyton/website/pages/cc.html. The advantage to this approach is that it provides the user with a tool for complex analysis of data. However, the disadvantage is that the process of fitting complex nonlinear models to data may be unfamiliar, and therefore the intricacies and pitfalls of the process must be appreciated if sensible information is to be gained. Furthermore, several quantitative models have been proposed for the analysis of lymphocyte data^{5,7-9,15}. Each of these models has different theoretical assumptions and each may

find uses in particular applications. However, we believe that the Cyton model is the most accurate and well-characterized description of lymphocyte proliferation.

The Cyton model differs from the precursor cohort method in the following ways. First, the variation in time to first division is described using a lognormal distribution instead of a Normal distribution. Second, deterministic parameters are not used to describe subsequent division and death times. Instead, the variation in these processes is described using lognormal distributions. And finally, the number of divisions that each cell progresses through can be regulated. Thus, the introduction of extra parameters not only increases the complexity of the Cyton model, but also gives a more accurate representation of the processes occurring in an *in vitro* culture. The theoretical basis of the Cyton model¹⁰ should be understood before it is utilized for data fitting.

Quantitative analysis is often used to examine the effect of some change in culture conditions on the underlying parameters of proliferation. In these situations, the Cyton Calculator can be used to test hypotheses. For instance, a basic situation might read as follows: data from graphical analysis might suggest that the division time is delayed as the concentration of 'stimulus A' is reduced in culture. Using the Cyton Calculator, the researcher would find an optimal solution for the highest concentration of 'stimulus A'. Subsequently, alternative fits for lower concentrations of the stimulus would be generated by changing only the length of the division time. If the rule holds true, the hypothesis is thus supported. If not, alternative parameters may need to be investigated. Examples of this approach are illustrated in systems studying regulation of T-cell growth by T-cell receptor stimulation and cytokines^{3,5,6}.

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