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Viewpoints

How the Change from FLM to FACS Affected Our Understanding of the G_1-Phase of the Cell Cycle

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ABSTRACT

The frequency of labeled mitoses (FLM) method for analyzing cell-cycle phases necessitates a determination of cell-cycle interdivision times and the absolute lengths of the cell-cycle phases. The change to flow sorting (FACS) analysis, a simpler, less labor intensive, and more rapid method, eliminated determinations of absolute phase times, yielding only percents of cells exhibiting particular DNA contents. Without an interdivision time value, conversion of these fractions into absolute phase lengths is not possible. This change in methodology has led to an alteration in how the cell cycle is viewed. The FLM method allowed the conclusion that G_1-phase variability resulted from constancy of S and G_2 phase lengths. In contrast, with FACS analysis, slow growing cells exhibiting a large fraction of cells with a G_1-phase amount of DNA appeared to be "arrested in G_1 phase". The loss of absolute phase length determinations has therefore led to the proposals of G_1-phase arrest, G_1-phase controls, restriction points, and G_0 phase. It is suggested that these G_1-phase controls and phenomena require a critical reevaluation in the light of an alternative cell-cycle model that does not require or postulate such G_1-phase controls.

After the original identification of cell-cycle phases by Howard and Pelc in the early 1950s, the next two decades brought forth a large number of measurements of cell-cycle phase lengths using the method of frequency of labeled mitoses (FLM). FLM is essentially the method use by Howard and Pelc.[1,2]

The FLM method is illustrated in Figure 1. Because this method is not now in common use, it is of interest to explain the method and the type of results obtained by this method. For FLM, growing cells are pulse-labeled with tritiated thymidine. After removal of label, growing cells are sampled at intervals and subjected to autoradiography. The fraction of mitotic cells that are radioactive is determined. As shown in Figure 1, there is an initial period with no labeled mitoses. This period gives the length, in absolute time, of the G_2 phase. Then there is a rise in labeled mitoses, a plateau, and a fall in labeled mitoses. The time interval of these labeled mitoses indicates the S-phase length. The G_1 phase length is determined from the $G_1 + G_2$ period—the period between the decrease in labeled mitoses and the subsequent increase in labeled mitoses—by subtracting the initially determined G_2 phase time. The total interdivision time (IDT) is determined by the time between the rise times of labeled mitoses.

The major generalization to come out of a large number of FLM analyses in the 1950–1970 period was that the G_1-phase is the most variable cell-cycle phase. In contrast, S and G_2 phases were relatively constant. Some studies demonstrated not only a large variability of the G_1 phase, but also a definite constancy of S and G_2 phases as interdivision time was varied. This observation of constant S and G_2 phases led to the postulation of an analogy between the bacterial and eukaryotic models for DNA replication.[3] In bacteria the constant C and D periods are analogous to the constant S and G_2 phases.

Examples of idealized FLM results are presented in Table 1. Consider a given cell type growing in four different growth conditions (A, B, C, D), each with a different interdivision time. If S and G_2 are invariant, then G_1 phase length is necessarily the difference between the interdivision time and the S phase length plus G_2 phase length. That is, $G_1 = IDT - (S + G_2)$. These results (Table 1) led to the conclusion that the G_1 phase length was determined by the overall growth rate or interdivision time, This reasoning led to a model of cell-cycle control that did not invoke any particular or unique events occurring in the G_1-phase.[4-8]

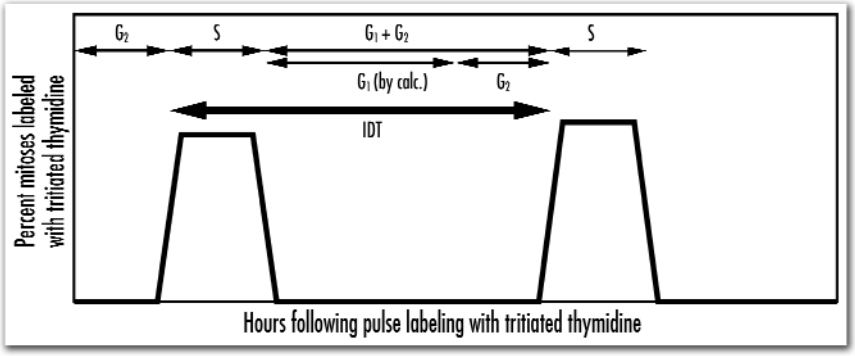

Figure 1 (left). Frequency of Labeled Mitoses (FLM) method for determination of cell-cycle phase lengths. To determine the cell-cycle phases using FLM, exponentially growing cells are pulse-labeled for a short period with tritiated thymidine. Only cells in S phase incorporate thymidine label. After removal of the exogenous label, cells are removed at intervals and analyzed by autoradiography. The fraction of mitotic cells with labeled DNA is determined. At the beginning of collection those cells in the G_2 phase at the time of pulse labeling produce dividing or mitotic cells over a time equivalent to the G_2 phase; there are no labeled mitoses during this initial period. After a period of time equal to the G_2 phase length, cells that were in S phase at the time of labeling now enter mitosis and one now sees labeled mitoses. There are labeled mitoses for a period of time equal to the length of an S phase. Then cells that were in the G_1 phase at the time of labeling produce mitotic cells and these are unlabeled. The period of unlabeled mitoses continues for a time equal to the sum of the G_1 phase and the G_2 phase. This is because the cells that were originally in G_2 phase at the time of pulse labeling now divide again. After the second G_2 phase period, cells again produce labeled mitoses. The length of the G_1 phase is determined by subtracting the G_2 phase length from the G_2+G_1 phase length. The total interdivision time (IDT) can be determined by the time from the first rise in labeled mitoses to the second rise in labeled mitoses. As shown here, the G_1, S, and G_2 phases are 50%, 25%, and 25% of the total IDT.

The wide-spread use of FLM, a very labor-intensive method, was eventually supplanted by FACS (fluorescence assisted cell sorting) analysis, a simpler, less labor-intensive, and more rapid method. In FACS analysis, cells are merely sampled from a growing cell culture and the total fraction of cells with different DNA contents is determined. An example of idealized results obtained by FACS analysis is presented in Figure 2. As seen in Figure 2, FACS analysis gives the fraction of cells with a G_1, S, or G_2/M phase amount of DNA. The absolute lengths of these phases are not given by FACS analysis. Determination of phase lengths requires knowledge of the total interdivision time. Multiplying the total interdivision time by the fractional amount of cells in each phase gives the absolute phase lengths. (The age distribution has been ignored in this simplified analysis.)

Idealized results for the same cultures (A, B, C, and D) as in Table 1 are presented in Table 2 as determined by FACS analysis. The absence of interdivision times leads to the description of the cultures by the conditions used to produce the different cell-cycle patterns. The data of Table 2 are actually the same as the data in Table 1, but the results are given as percentages of the cells in different phases. As the serum concentration is decreased (a classic method for cell growth "arrest") more cells have a G_1-phase amount of DNA. Without knowing the interdivision times, it has been generally concluded that the increase in cells with a G_1-phase amount of DNA indicates that the cells are arrested at a particular point or control element in the G_1 phase.

FACS analysis therefore gives a very different picture of the cell cycle than FLM methodology. The constancy of S and G_2 phases is not apparent with FACS analysis. The dominant result with FACS analysis is the increase in the fraction of cells with a G_1-phase amount of DNA.

The results in Table 2 for slow growing cells (low, lower, or very low serum) have been interpreted as indicating arrest of cells in the G_1 phase or arrest "at a point in G_1 phase" (e.g., the restriction point), or entrance of the cells into an "out-of-cycle-phase", G_0. But it is important to emphasize that it is not postulated that the cells analyzed in Tables 1 and 2 are arrested. The cells described in Tables

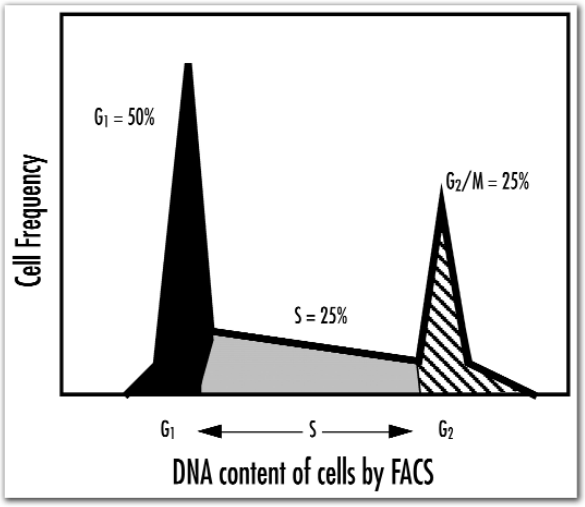

Figure 2. Flow-cytometric (FACS) determination of fraction of cells with particular DNA contents. For FACS analysis, exponentially growing cells are labeled with a fluorophore that reflects the DNA content of cells. As each cell is assayed, the DNA contents are measured. As shown here, for cells with 50% G_1 phase cells, 25% S phase cells, and 25% G_2 phase cells, one can measure the total number of cells in the specific portions of the DNA contents. (N. B. For simplification, no account has been made of the age distribution of cells.)

Table 1 **FLM Analysis of Cell Cycle**

	Interdivision Time	G_1[a]	S[a]	G_2[a]
A	20 hours	10	5	5
B	100 hours	90	5	5
C	1,000 hours	990	5	5
D	10,000 hours	9,990	5	5

[a]Phase lengths in hours

Table 2 **FACS Analysis of Cell Cycle**

	Treatment	G_1[a]	S[a]	G_2[a]
A	Full serum	50	25	25
B	Low serum	90	5	5
C	Lower serum	99	0.5	0.5
D	Very low serum	99.9	0.05	0.05

[a]Percent of cells observed with specific DNA contents.

1 and 2 are assumed to be growing steadily but at different rates of growth. As shown in Table 1, the cells are merely growing slowly as the serum as reduced (described in Table 2). The imputation of cell "arrest" is merely due to the experimental difficulty of following cell growth over many hours and determining whether or not cells are growing slowly.

To be precise, the concepts of G_0 and the restriction point and G_1-phase arrest points pre-dated the development of FACS analysis. What has happened, however, is that the facile use of this instrumentation has led to appearance of a large amount of support for these G_1-phase control elements. The point made here is that the ubiquity of FACS analysis has made it appear as though a large amount of experimental evidence independently supports these G_1-phase arrest phenomena. As shown in Tables 1 and 2, this is not the case.

We see that the advent of a the newer, simpler, more rapid, and quantitative method, FACS analysis, displacing the FLM method, has led to omission of a major point, the constancy and relative invariance of the S and G_2 phases. Thus, FACS analysis can give an incorrect view of the cell cycle. As shown for bacteria, the constancy of DNA replication time (S phase in eukaryotes, C period in bacteria) and the constancy of the period between termination of DNA replication and cell division (G_2 in eukaryotes, D period in bacteria) leads to a model of cell-cycle regulation that does not postulate the importance of passage through G_1-phase specific events, restriction points, or checkpoints. The experimental and theoretical support for this view of cell cycle control has been reviewed.[4-7]

Instead of envisioning specific G_1-phase controls, this alternative model proposes that control of cell-cycle events is based on a continuous accumulation occurring in all phases of the cell cycle. The variable G_1 phase is a consequence of variation in growth rate throughout the cell cycle. The variable G_1 phase is not a consequence of how rapidly or slowly specific G_1-phase events are carried out. FACS analysis, in contrast to FLM analysis, leads to the idea of G_1-phase arrest, G_1-phase control elements, and other G_1-phase phenomena. The current model of the cell cycle should therefore be reevaluated and reconsidered in the light of this historical analysis.

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