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Mammalian cells are not synchronized in G_1-phase by starvation or inhibition: considerations of the fundamental concept of G_1-phase synchronization

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Abstract. Synchronization of mammalian cells by starvation-refeeding or by inhibition-release are among the most commonly used techniques for division cycle analysis. An alternative analysis—in the form of a *Gedanken* or thought experiment—is presented, casting doubt on the utility of this synchronization method. Arresting cell growth produces a culture where all cells contain a G_1 amount of DNA. However, these cells are not arrested at a particular point in the G_1-phase. Analysis of 'G_1 arrested cells' suggests that, upon resumption of growth, the cells are not synchronized.

I wish to reanalyse the major experimental approach for the analysis of the mammalian cell cycle—the synchronization of mammalian cells in the G_1-phase of the division cycle using starvation or inhibition techniques. This method is arguably the most prominent, most utilized, and most influential experimental approach for analysing the cell cycle. Although one could cite a large number of papers using the starvation/synchrony method, perhaps two examples may suffice. In one paper (Pellegata *et al.* 1996), the methods section states that 'Synchronized cell populations were obtained by release from confluence/serum deprivation-induced G_0 arrest'. In another paper (Lindeman *et al.* 1997), 'cells were arrested in G_0 by serum starvation for 72 h and then released into the cycle by refeeding with 10% serum for 24 h'. An enormous number of additional examples may be easily found throughout the literature.

I will evaluate the block-and-release synchronization method by presenting a counter-example (or perhaps it should be called a counter-analysis) which leads to an alternative explanation of how cells resume growth after arrest. This analysis is presented in the form of a *Gedanken* or thought experiment. An idealized situation is presented in which it is shown that while cells may be starved such that one property is uniform in all cells (i.e. all cells may have the same DNA content), these cells are not necessarily synchronized.

Starvation synchronization

The method of starvation or growth arrest synchronization is illustrated in Figure 1. Consider a cell population with cells in all phases of the cell cycle. Flow-cytometric analysis of this population (at the upper right) shows a peak for G_1 cells, a broader central distribution for

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S-phase cells, and another peak for G_2-phase cells (upper panel). When cells are starved (or growth is inhibited), usually for a long time, the resulting cells have a single DNA peak. As the DNA content of these growth arrested cells is similar to the DNA content of cells in the G_1-phase of the division cycle (that is, a G_1-amount of DNA), the cells are proposed to be arrested in the G_1-phase of the division cycle (middle panel). (Some investigators write about the G_0-phase interchangeably with the G_1-phase; the analysis presented here applies equally to either terminology.

When cells are re-fed and allowed to resume growth, the G_1-arrested cells are believed to pass through the stages of the G_1-phase that occur after the arrest point. The cells are then believed to pass synchronously through the S and G_2-phases to the final synchronous division (Figure 1, lower panel). Ideally, if the cells were synchronized, and if interdivision variation was not too great, there would be a second cycle of progression through a G_1-phase, an S-phase, a G_2-phase and an M-phase.

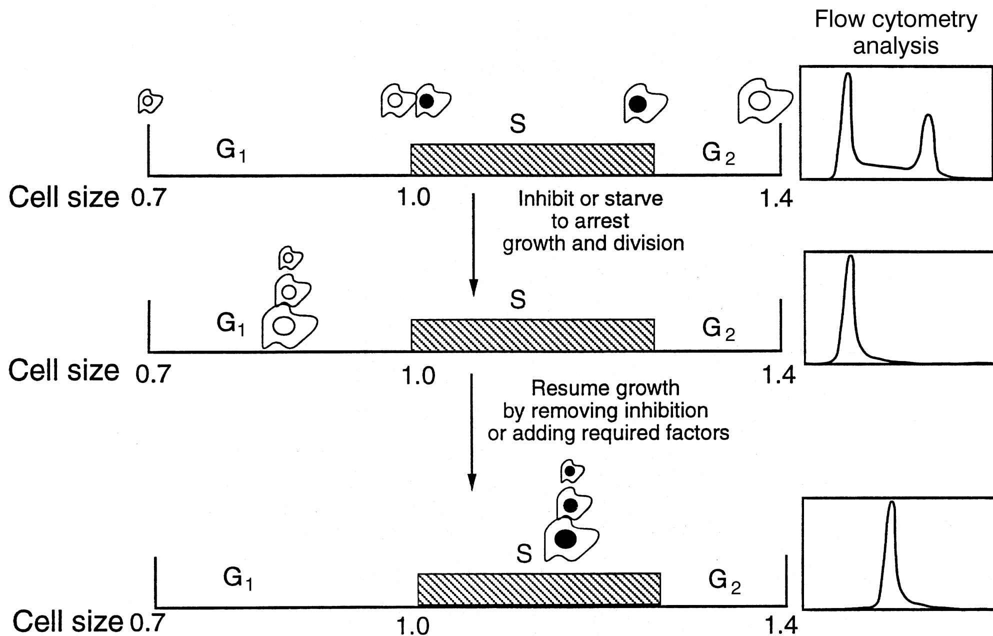

Figure 1. Starvation synchronization—the current view. Consider cells growing between sizes of approximately 0.7 and 1.4. There are cells in all phases of the division cycle as indicated by the idealized flow-cytometer analysis at the upper right. DNA synthesis initiates in the middle of the division cycle, at a size of approximately 1.0. Cells in S-phase (synthesizing DNA) are represented by a filled nucleus. After starvation (centre panel), cells all have a G_1-amount of DNA. It is generally believed that cells are now arrested at a particular point in the G_1 phase called, among other names, the 'restriction point'. The observed flow cytometric pattern is illustrated (centre right) showing that all cells have a G_1 or a 2n amount of DNA. Upon restimulation of growth, the cells are believed to pass synchronously through the events of the division cycle. The cells pass from the G_1 point of arrest through the S-phase, and then through the G_2 and M-phases. The cells are illustrated (lower panel) passing synchronously through the S-phase of the cell cycle.

Alternative explanation of starvation synchronization

Once more, consider a growing population with cells in all phases of the division cycle (Figure 2, upper panel). For simplicity, concentrate on two cells, one just before (cell A), and one just after (cell B) the start of S-phase. Since initiation of DNA synthesis in this *Gedanken* experiment starts at cell size 1.0, these two cells have almost the same size; for example, cell A is size 0.9999, and cell B is size 1.0001. (Parenthetically, it should be noted that in a real culture there is a great deal of variation in the size of newborn cells and dividing cells, as well as the size of cells at the start of DNA synthesis; the presentation of an idealized culture with no size variations will emphasize the theoretical problem with starvation synchronization. Of course, any variation in cell sizes or variation in normal interdivision times will only exacerbate the problem of synchronizing cells by starvation.) These two cells have a negligible size difference because the G_1-phase cell is only slightly younger than the cell that just started S-phase. Now consider what happens when these two cells (and all other cells in the culture)

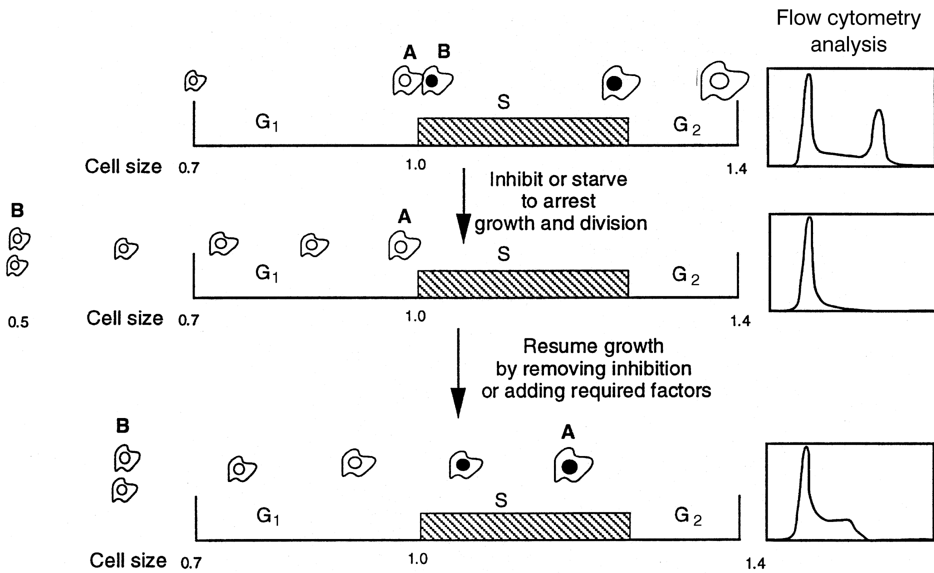

Figure 2. Starvation synchronization—the alternative view. Consider cells growing between sizes of approximately 0.7 and 1.4. There are cells in all phases of the division cycle as indicated by the idealized flow-cytometer analysis at the right (upper panel). DNA synthesis, illustrated by the filled nucleus, initiates in the middle of the division cycle, at a size of approximately 1.0. Although cells in all phases of the cycle are illustrated, consider the two cells of approximately size 1.0, one just before, and one just after, the start of S-phase. The cell with a G_1-DNA content does not initiate DNA synthesis and remains a cell with a G_1-DNA content. The S-phase cell, on the other hand, proceeds through division and produces two cells of size 0.5 (centre panel). This is because the mass does not increase during the period of inhibition, and when the cells coming from the early S-phase cells divide, the resulting daughter cells are half the size of the G_1 cells that did not divide. The cells produced after starvation therefore differ in size by a factor of two. All of the other cells in the original culture are of sizes intermediate between size 0.5 and 1.0, and all have a G_1-amount of DNA. They are not arrested or synchronized at a particular point in the G_1-phase of the cycle following starvation, for the cells are actually approximately one doubling time apart. After resumption of growth is allowed (lower panel), the first cells to initiate DNA synthesis (enter S-phase) are the cells that were just about to enter S-phase but were inhibited by the starvation or inhibition protocol. The cells that arose by division must grow for one extra doubling time before they initiate DNA synthesis. Initiation of DNA synthesis is not synchronized, being spread over one doubling time.

are subjected to starvation or growth inhibition to induce G_1 arrest. Assume that serum starved cells or growth inhibited cells are inhibited in mass synthesis. That there is an inhibition of mass synthesis during such starvation protocols is clear from the fact that G_1-arrested cells do not continue growing to produce large cells. If mass synthesis were allowed to continue without cessation, then in arrested cells that did not divide the size of the cells would increase without bound. In this same vein, it should be noted that if mass synthesis did not abruptly stop, but only slowly came to a half, all of the arguments made here would still apply, but the cessation of initiation and the cessation of cell division would be delayed until mass synthesis stopped. The smaller cell (A), still in G_1-phase, does not initiate DNA synthesis as growth to size 1.0 is required for initiation of DNA synthesis; it remains a cell with a G_1-amount of DNA. In contrast, the S-phase cell (B) completes DNA synthesis and undergoes a mitosis and a cell division to produce two daughter cells. This, of course, is required for all cells to have a G_1 amount of DNA. What is the DNA content of these two daughter cells (B) in the centre panel? They have a G_1-DNA content. What is the size of these two daughter cells? Since cell size did not increase due to starvation during the completion of DNA replication and mitosis (as assumed above), the two daughter cells have a size of approximately 0.5. All other cells in the growth arrested culture have sizes between $\sim$0.5 and $\sim$1.0. Flow cytometric analysis reveals that all the cells have a common DNA content, equivalent to the G_1 amount of DNA (Figure 2, centre right). The arrested cells are not all the same size, however, and the smallest ones (B) are even smaller than the smallest cells of the normally growing culture illustrated at the top of Figure 2. Thus, in a normal culture the minimum cell size as illustrated in this example is size 0.7. It is not important that this be the precise size of the newborn cell, for all that needs to be seen is that it is not possible, for a culture in which the cells that initiate DNA replication are size 1.0, to have newborn cells of size 0.5. This is because the dividing cells will be of a size somewhat greater than 1.0 since mass synthesis will occur between initiation of DNA synthesis and cell division, and thus the dividing cell will have to be of a size greater than 1.0. For this reason, in the normal culture the newborn cells must have a size greater than 0.5.

We now have a collection of cells, all with a G_1 DNA content. Are these cells 'in G_1?' A distinction should be made between G_1 cells found in exponentially growing cultures and cells that have as one of their properties a G_1 DNA content. This distinction leads to the paradox that *cells may have a G_1 DNA content and not be 'in G_1'.* These starved cells all have a G_1 amount of DNA although many have a non-G_1 amount of cell mass (i.e. an amount of cell mass that is less than the cell mass of the smallest cells in the normal, unperturbed culture. To explain this paradox, as can be seen in Figure 2, at least some of the starved cells have a cell size that is smaller (i.e. 0.5) than the smallest cells in the steady-state, exponentially growing culture (i.e. 0.7). The cells that are arrested with a G_1-amount of DNA are not like the normal G_1 cells in a growing population. They are usually called 'cells arrested in G_1', but it is more precise to refer to these cells as 'cells with a G_1 DNA content'. This is a description of what is observed, and does not propose that the cells are in the G_1-phase of the division cycle. The more general point is that after starvation the cells with a G_1 amount of DNA have a variety of cell sizes with the same cell size variation—the cell sizes vary over a factor of 2 (ignoring normal cell size variability)—as in the original culture.

Continuing with our *Gedanken* experiment, allow growth to resume. Which cell starts DNA synthesis first? Since the cell arrested without further DNA synthesis and division (cell A) is of size 0.9999, it starts DNA replication (i.e. enters S-phase) almost immediately as very little synthesis is required to reach cell size 1.0. How long does it take the cells of size 0.5 (cells labelled B) to reach size 1.0? One full doubling time. Cells arrested with a G_1 amount

of DNA are *not* synchronized, as they enter S-phase over a period that is the same as the normal mass doubling time of the culture. Cells of intermediate starvation size are spread out between cells B and A, and enter S-phase at different times. If a culture was synchronized, a particular cell cycle event (such as initiation of S-phase) should take place over a time period that is a relatively small fraction of the normal doubling time of the cells. Initiation of DNA synthesis occurs over a time period equal to one doubling time of the culture, and thus the cells are not synchronized.

The tacit assumption in this analysis, which can now be made explicit, is that the initiation of the S phase occurs when there is an accumulation of cell mass to size 1.0. At a molecular level, initiation of DNA synthesis may be due to some specific material that is made along with cell mass. But the important point is that whatever is the ultimate initiator of DNA replication, this material is *not necessarily* made in a cell cycle-specific manner. (I do realize that the current view of the mammalian cell cycle postulates that initiation of S-phase, or exit from G_1-phase is governed by syntheses occurring at specific points of the division cycle. I do not make this assumption, and the very point of this paper is to note that essentially all of the experiments that have been put forward to demonstrate mammalian cell-cycle specific syntheses are based on starvation-release experiments or similar perturbing methods. The arguments made here take issue with the very heart of the current view of the division cycle (Cooper 1991).) All that needs to be assumed is that when cells reach size 1.0 they have enough of some material to initiate S-phase, and pass through the S, G_2 and M-phases. No cell cycle-specific syntheses are assumed or required. No G_1-specific arrest points are assumed or required. Even if there were no cell cycle-specific events, and even if cells were not synchronized at a point in the division cycle, one would obtain cells after starvation or growth inhibition that have a G_1 amount of DNA. But as illustrated in Figure 2, these cells are not arrested at a particular point in the G_1-phase of the division cycle. And these cells do not exhibit synchronized growth when growth is allowed to resume.

Experimental support

Experimental support for the alternative analysis presents an amusing conundrum. If I were to perform a starvation/refeeding experiment and not get synchrony, I could be accused of not doing the experiment well enough, or enough times, or with enough care, to get the 'right' result. Doing my own experiments involves a kind of Catch-22; failure to synchronize cells would not be accepted as proof of the alternative analysis. What is needed are unbiased experiments, or even experiments biased toward synchrony and cell-cycle arrest at a particular point. If such experiments indicate that no G_1 specific arrest point exists, and that cells are not synchronized upon regrowth after arrest, then the point made is much stronger.

The earliest work on arrest at a particular point in the G_1-phase, the classical experiments on the restriction, or R, point, did not produce a synchronized culture. To briefly recapitulate, the 'restriction point' was proposed to exist when it was observed that starvation for different compounds led to a common time until DNA synthesis resumed following refeeding (Pardee 1974). The restriction point model proposed that the arrested cells, no matter what the conditions of arrest, stopped at the same point prior to the start of S-phase. In this experiment, however, the resumption of DNA synthesis was determined by measuring the time until DNA synthesis started. Only the small subset of early starting cells were used to measure the time of reinitiation of DNA synthesis. However, if one considered the entire population, there was a significant spread in time for cells to enter S-phase. In the original restriction point paper (Pardee 1974), it was reported that 'different cells begin thymidine

incorporation at different times. Thus, measurement of the time of initiation of DNA synthesis by a cell population depends upon the behaviour of an early initiating subclass of the population. We can only conclude that this subclass is at the same point in different quiescent cultures'. The restriction point was therefore defined by a small subset of the total population. The notion of a specific arrest point is therefore dependent upon the behaviour of a few cells (i.e. those cells that incorporate thymidine earliest after refeeding), and not the majority of the population. The majority of the cells released from the quiescent state start DNA synthesis (thymidine incorporation) at different times, just as proposed here and illustrated in Figure 2. Since all of the starvation regimens in the restriction point paper specifically affected protein synthesis, the finding of an identical time until reinitiation occurred is expected, and is not supportive of synchrony or supportive of a specific arrest point. Most important, all of the different starvation regimens did not lead to a sharp timing of a cell-cycle event such as the start of S-phase; the cells were not synchronized.

The lack of synchrony of starvation-synchronized cells is strikingly illustrated in a more recent paper. Cells were starved and released from starvation in order to synchronize the cells and to study syntheses during the division cycle (Di Matteo et al. 1995). As it was phrased, '...we used NIH/3T3 fibroblasts that can be brought to the resting state by serum withdrawal; the proliferation block can then be released by supplementing serum again, thus stimulating cells to synchronously reenter the cycle' (Di Matteo *et al.* 1995). These experiments were specifically aimed at synchronizing cells. The results of such starvation refeeding is illustrated in Figure 3 (redrawing of Figure 5B of Di Matteo *et al.* 1995). As seen in Figure 3, removal of serum for 36 h produced a cell population that did not move synchronously into the S-phase of the division cycle as predicted by the G_1 arrest model of Figure 1. Rather, some cells entered the S-phase while others remained with a G_1 amount of DNA as predicted by the alternative analysis in Figure 2. The flow cytometric measurements of DNA content of cells following starvation/refeeding indicates that the cells do not move as a cohort

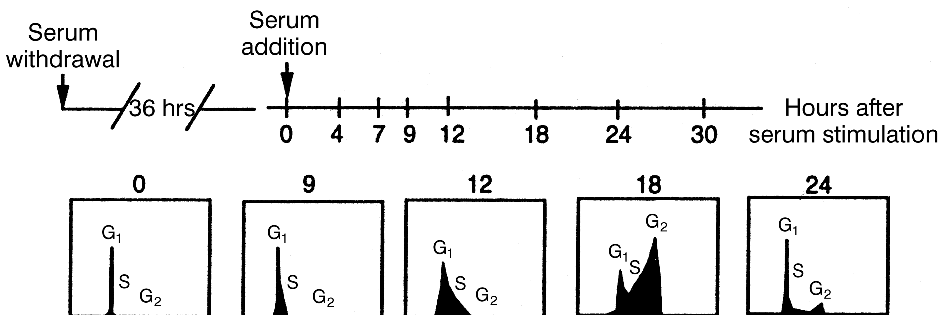

Figure 3. DNA contents of cells released from serum starvation. This is the redrawn data of Figure 5B from De Matteo *et al.* (1995). For the record, Mouse NIH/3T3 fibroblasts (ATCC CRL 1658) were grown in DMEM supplemented with 10% FCS in a 5% CO_2 atmosphere. For synchronization, confluent cells were maintained in a medium containing 0.5% FCS. After 36 h FCS was added to 10% and cells were harvested at regular intervals and the DNA content was assayed by FACS. The DNA content was monitored by harvesting the cells using trypsinization, resuspending the cells in 5:1:4 PBS–acetone–methanol and incubating the cells with 10 μg/ml RNase for 5 min on ice. Propidium iodide was then added to a concentration of 50 μg/ml, and the samples were incubated in the dark for 30 min. Samples were then analysed in a FACStar Plus cytofluorimeter using the Multicycle software to determine the DNA content. At least 10 000 cells were analysed per sample. The original figure was copied to a transparency, the transparency was placed over a computer screen, and the DNA distributions were traced from the transparency.

through the division cycle. The observed pattern is more supportive of lack of synchrony (Figure 2) than synchrony (Figure 1).

After 24 h the DNA distribution was that of a normal culture. If cells were truly synchronized, one would expect (as noted above) a movement through a succession of flow cytometric diagrams where cells were primarily G_1-phase, then S-phase, then G_2-phase, and then G_1, S, and G_2-phases again for at least one more cycle. The observed steady-state distribution of cells after 24 h implies that cells are not arrested at a particular point and are not synchronized upon release from serum starvation.

The proposal made here uses a very clear definition of what it means to be synchronized. A culture is synchronized when all of the cells pass through successive phases of the division cycle at essentially the same time. Of course there would be normal variation, and it is not proposed that cells have to all start DNA replication over a period of minutes or even an hour. But one must distinguish between the occurrence of one event over a narrow period of time and the synchronization of a culture. If a starved culture truly reflects the normal cells in a particular phase of a growing culture, then the starved culture, upon refeeding, would not only start DNA replication over a narrow period of time but would also divide over a narrow period of time. Merely using one criterion is not enough and, even more importantly, a second round of division with a normal division cycle must also be demonstrated. This has not been shown to exist in mammalian cells.

I am unware of any experiments using starvation to synchronize mammalian cells that presents an analysis of a second round of synchronized growth and division. And I am unaware of any starvation or inhibition synchrony experiments that show two cycles of synchronized cell division. Cell division synchrony is a defining characteristic of successful synchronization (Cooper 1991).

With regard to the analysis of cell-cycle events or cell-cycle syntheses upon resumption of growth, first division cycle experiments are subject to the legitimate criticism that any observed phenomenon may be an artefact of the starvation/refeeding procedure. An example of this type of artefact has been described for the synthesis of c-*myc* (Cooper 1989).

This analysis is independent of whether or not there is an observed delay in the start of DNA synthesis upon release of starvation or inhibition. Such a delay only makes it appear as though the cells are synchronized. The resumption of DNA synthesis in different cells takes place over a wide range of times, and so the cells are not synchronized nor are cells arrested at a particular point in the division cycle.

Implications of this analysis

What is a G_1 cell? The problem is one of both vocabulary and experiment. For exponentially growing cells in an unlimited medium, the answer is simple: G_1 cells are cells with a G_1, or 2n, DNA content. Temporally, they are cells between mitosis and the start of the S-phase. When S-phase starts, the amount of DNA immediately increases and the cells do not have a 2n DNA content; the cells are now in S-phase.

In the analysis here, we show that the G_1-phase entails more than DNA content. True, G_1 cells are cells with a 2n amount of DNA. G_1-phase cells, during normal growth, are between mitosis and S-phase. There is more to a G_1 cell, however, than its DNA content or DNA configuration. G_1 cells have an additional property that distinguishes them from cells in other parts of the cycle. Between cell divisions there is a continuous variation in cell size; G_1 cells are smaller, on the average, than cells in S, and even smaller than the cells in G_2. And cells in the early part of G_1-phase are smaller than cells in the later part of G_1-phase. We may postulate that there is a continuous accumulation of some triggering substance that will

initiate S-phase when enough has accumulated. This material may be synthesized throughout the division cycle, and thus be cell-cycle independent rather than cell-cycle specific. According to this view, in each phase of the cell cycle there is an amount of S-phase triggering substance characteristic of that particular phase. Starvation prevents accumulation of this triggering material, and cells are arrested with a G_1 amount of DNA. But note that these cells are not arrested at a specific point or event in the cell cycle, because the arrested cells all have different amounts of the triggering material. That is why cells, upon being released from starvation, initiate DNA synthesis over a long period of time. Cells with more triggering substance initiate earlier, and the cells with the least triggering substance, initiate later.

This description of growth during the cell cycle leads inevitably to a reexamination of the generally accepted view of the starvation/synchronization method. To be rigorous and precise, it is not argued here that it is impossible for the starvation/synchrony method to work; one cannot prove experimentally that it will not work. To generalize, one cannot experimentally prove a universal negative. Nevertheless, to my knowledge, this alternative explanation described here has not been strongly considered. The starvation/refeeding synchronization method has been a fundamental and oft-used experiment for cell cycle analysis. A close reading of experimental results using this method indicates that cells proposed to be synchronized are not, or may not be, synchronized. The implication of this critique is that one must be wary of starvation experiments that are used to synchronize cells. If the analysis presented here is correct, then we must reevaluate a large part of the experimental work on specific cell-cycle-dependent synthetic processes in the mammalian division cycle.

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