On the Persistence of Forcing Synchronization Methodology

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Theoretical as well as experimental considerations indicate that forcing methods cannot synchronize cells. Nevertheless, forcing methods of eukaryotic cell synchronization are the most widely used approaches to cell-cycle analysis. Why, despite experimental and theoretical critiques, are these methods still so widely used? Reasons for the persistence of forcing synchronization methods are explored.

It is generally believed that it is possible to synchronize cells by treating growing cells in a “forcing” manner. When cells of various cell-cycle ages are treated identically and growth arrested, it is presumed that they can be forced into a condition where the cells are of a common cell-cycle age. It is further believed that these cells can form the start of a synchronized culture. One common and often-described experiment involves placing growing mammalian cells in a low-serum medium leading to growth arrest. The arrested cells are assumed to enter a G0 or a G0/G1 phase, or to arrest at a particular restriction point within the cell cycle. Upon resumption of growth by addition of normal serum concentrations, the cells are believed to move as a synchronized cohort through the cell cycle. Other treatments such as hydroxyurea to inhibit DNA replication, nocodazole to

inhibit mitosis, or mimosine inhibition, are also proposed as synchronizing agents. Even the cholesterol-lowering drug lovastatin has been suggested as a synchronizing agent. Yeast cells have been synchronized by treatment with -factor, which is proposed to arrest cells “in the G1-phase”. Yeast cells have also been “synchronized” by raising the temperature of mutants with temperature-sensitive defects. The general idea uniting these diverse methods is the assumption that cells can be arrested at some particular point in the cell cycle. Upon release of the arrest condition, the “synchronized” cells are proposed to grow as a synchronized cohort, passing as a relatively uniform group through the sequential phases of the cell cycle.

Forcing synchronization methods have been used for many years to study events during the cell cycle. These methods are accepted as valid approaches to cell-cycle analysis; hundreds to thousands of papers have used these methods. The results from these studies are published and reviewed in respectable journals. These methods are summarized in major cell biology textbooks. A large body of cell-cycle analysis is based on the use of forcing synchronization methods. Anyone entering the field of the cell cycle would see numerous reviews about how some forcing method can be used to “synchronize” cells. Because of the widespread use of these methods to synchronize cells, newcomers to cell-cycle studies readily apply these forcing methods to the study of their particular enzyme or protein.

But it has been proposed that it is theoretically impossible to synchronize cells by any forcing method (1-3). This pronouncement, unsupportive of the current and widely used forcing methodologies, is generally and widely ignored. The field continues to use methods that, in theory and in practice, do not synchronize cells.

I wish to briefly review the theoretical argument against forcing synchronization. I will then buttress this theoretical analysis with a review of the experimental work

demonstrating that forcing synchronization methods do not work and that the use of these methods must be re-evaluated. If the forcing methods proposed to synchronize cells do not synchronize cells, then any results regarding cell-cycle control that are derived from these experiments must be re-examined.

The argument against forcing methods is simple(1-6). Exponentially growing cells have varied DNA contents and varied cell sizes. Cell size varies over a factor of at least two, as the newborn cells are half the size of the dividing cells at the end of the cell cycle. Cells of intermediate cell-cycle ages have intermediate sizes. In order to produce a synchronized culture, one must align cells so their DNA content is uniform. There must also be a narrowing of cell size so the initial cells are similar to the size of cells at some particular cell age. A detailed analysis of the three fundamental classes of synchronization methods, arrest of mass growth, arrest of DNA replication, and arrest of mitosis, indicate that none of these methods, in theory, can lead to a truly synchronized culture (1). The inability to synchronize cells results from the fact that none of these methods produce a narrowing of cell size distribution. Because inhibition of mass increase does not lead to cells stopping growth at a particular cell size, and because inhibition of DNA synthesis or mitosis does not arrest mass increase, there is no narrowing of cell size distributions.

Why must the initial cells of a synchronized culture have a narrow cell size distribution reflecting some cell-cycle age during unrestricted growth? There are two answers to this question. Assume that there is a progression of events during the cell cycle, and that these events occur at different cell ages and thus at different cell sizes. If the size distribution is not narrowed, the initial cells after forcing treatment are in different parts of the progression of cell-cycle events—even though they may all have a common DNA content. Alternatively, assume that growing cells initiate DNA replication

at some cell-cycle age and at some particular cell size. If achievement of a certain cell size is a critical control system, then a group of "synchronized" cells with varied sizes will reach the initiation size at different times. This leads to an absence of synchrony.

Theoretical arguments against synchronization by forcing methods have been strongly supported by much experimental evidence. A reanalysis of the forced synchronization of mammalian cells (7) showed that the evidence for synchronization actually indicated that the cells were not synchronized (2). An analysis (8) of microarray studies of cells synchronized by a double-thymidine block (9) indicated that the cells were, in fact, not synchronized. A demonstration of the lack of synchronization of cells by a forcing treatment is the time-lapse, videographic, analysis of cells treated with lovastatin (10). In contrast to the proposal that lovastatin is a synchronizing agent (11) it was shown by direct examination of cell division patterns that cells are not synchronized by lovastatin treatment (10). In addition, the reanalysis of lovastatin inhibition (10) suggested that the original data on synchronization (11) was consistent with a lack of synchrony. In addition, data showing that lovastatin-treated cells are arrested in the G1-phase of the cell cycle (12, 13) has been reinterpreted concluding that the cells were not actually arrested in any particular phase of the cell cycle (10). Other laboratories have also presented data that indicate that there is no synchronization using lovastatin (14). Experiments studying cells placed in a "G0 phase" from which cells are proposed to emerge as a synchronized cohort (15-17), actually support the idea that such cells are not synchronized (5, 6, 18).

Additional data supports the idea that forcing methods do not synchronize cells. For example, a study of the cell synchronization agents compactin, ciclopiroxolamine, mimosine, aphidicolin, ALLN, and colcemid indicated that it was not clear that the methods actually synchronized cells. It was concluded that the experiments demonstrated

that forcing synchrony methods differ with respect to their impact on cell cycle organization and do not synchronize cells (19).

Finally, the original work on restriction point arrest (20, 21), the classic ancestor of all arrest methods for synchronization, supports the suggestion that cells are neither arrested at a particular point in the G1 phase nor synchronized after release (22).

Why then, despite theoretical and experimental support for the proposal that forcing methods do not synchronize cells, does the use of forcing synchronization methods still hold sway over the field of cell biology and cell-cycle studies?

One answer to this question notes the absence of explicit criteria to define a synchronized culture. The ease of Fluorescence assisted cell sorting (FACS) analysis to determine the DNA distribution of a cell population has led to the straightforward demonstration that growth arrested cells accumulate with a G1-phase amount of DNA. It has been pointed out (1-3) that a distinction must be made between “arrest with a G1-phase amount of DNA” and “arrest at a point in G1 phase.” Analyses of arrest methods indicate that there is no narrowing of cell size distributions even though cells may all have a common DNA content (1). FACS analysis, rather than being a boon to cell-cycle analysis, may actually be a bane, as it allows an easy acceptance of one particular aspect of a cell (e.g., DNA content) as a surrogate measure of cell age. The DNA content is taken as a measure that the cells are of the same age, and therefore synchronized. But this is not necessarily the correct conclusion. Cells may have a common DNA content (e.g., a G1-phase amount of DNA) but the cells may not be representative of G1-phase cells or cells of any particular cell-cycle age. When some DNA analyses during cell growth are presented to support a presumed synchronized

culture, the actual support for synchronization is not there; sometimes the data indicate the cells are actually not synchronized (7, 23, 24).

The ease of FACS analysis in defining synchrony contrasts with the difficulty of measuring synchronized cell divisions, the most stringent definition of synchrony. Demonstrating that mammalian cells have synchronized divisions is labor intensive. Points must be taken every hour or two, for up to fifty hours. Also, the expected cell number change at a synchronized division is expected to fluctuate only over a factor of two. For adherent cells that require numerous steps between cell harvesting and counting, there is a large cell number variation that precludes a precise demonstration of synchronized divisions. The data must show that cells divide over a relatively short span of time, and points between these rises should show clear plateaus of non-division. Cell division analysis of synchronized cells is hardly ever done.

One argument raised against using the criterion of synchronous divisions as a measure of synchronization is the widespread belief that even if cells were truly synchronized, one would not get good division synchrony because of the rapid decay of synchrony. This argument eliminates the necessity to show two cell cycles of synchronous growth. With the development of the eukaryotic “baby machine” by Charles Helmstetter, this argument has been shown to be incorrect. The baby machine produces well-synchronized, normal, unperturbed cells (25-27). Not only do the cells produced by membrane-elution exhibit at least three clear synchronized divisions, but they also exhibit the proper size and DNA contents during synchronized growth (25-27)., satisfying the basic and stringent criteria for synchronized growth. If cells produced by forced synchronization are truly synchronized, then one should be able to see synchronized divisions. The absence of division synchrony is evidence that the cells are not synchronized.

Published data on the eukaryotic membrane-elution method indicates that not only do cells have the proper DNA content as they move through the cell cycle, but the eluted cells also show the expected variation in cell size paralleling the variation in DNA content. No other synchronization method has been shown to fit these rigorous criteria for synchronization. Not only do the cell numbers in the synchronized cultures eluted from the membrane follow a synchronized pattern without connecting lines to emphasize synchrony, but the DNA contents, and cell size distributions, repeat over at least three cycles (25, 26).

The synchronized cultures produced by the membrane-elution method are a new "gold standard" against which other methods must be measured. If other methods were compared to the membrane-elution method, the inability of these methods to produce a synchronized culture would be evident.

Supporting forcing synchronization methods are the many researchers who want to study and analyse their particular system as a function of the cell cycle. There is something about the cell cycle that draws researchers to ask, "How does my enzyme, or the expression of my gene, vary during the cell cycle?" Investigators entering the field of cell-cycle studies adopt these widely used forcing methods. This may be due to the simple fact that one hasn't time to re-examine the experiments and forcing methods that have been in use for almost three decades. The papers produced by new entrants to cell-cycle studies often lack experimental proof (e.g., DNA contents, cell number variation, size distributions), that the cells being analysed are truly synchronized. Even when data is presented to demonstrate that cells are synchronized, a reassessment of the published data indicates that the cells may not actually be synchronized (2, 7, 10, 14).

Another reason these forcing methods are used is that they are simple. Cells are grown, cells are placed for a number of hours in a particular arrest condition, the arrest condition is reversed, and the released cells are allowed to grow. This simple experimental design, coupled with the ease of FACS analysis for assaying DNA contents, have given rise to the idea that it is easy to synchronize cells by forcing treatments. But merely aligning cells with a common DNA content (e.g., “arrest with a G1-phase amount of DNA”, indicates “arrest at a point in the G1 phase”) does not mean that cells are truly synchronized and representative of cells during the normal cell cycle. Ease of experimentation does not mean that the method is correct.

Another problem is the group aspect of cell-cycle research. It is extremely difficult to break away from the prevalent method of doing things. It is easy to say “I am working on G0 cells”, rather than to say “I am studying cells starved in low serum for 40 hours, producing cells with a G1-phase amount of DNA (which I believe are synchronized cells), and then I study these cells by adding serum and taking samples every hour, although I have yet to show that the cells are truly synchronized and have synchronized divisions.”

Characterizing these explanations for the persistence of forcing synchronization is the extremely large number of papers using forcing methods. The response to critiques of forcing synchronization is usually in the form of a question: “How can a few experiments, and a few theoretical arguments, criticizing forcing synchronization methods compare with the hundreds and probably thousands of papers reporting that forcing methods do synchronize cells?”

A rigorous adherence to scientific principles suggests that those who wish to use forcing methods respond to the theoretical and experimental critiques raised here.

Arguments based on the number of papers using forcing synchronization do not answer the concerns raised regarding these methods. Experiments using forcing synchronization should show that the cells have synchronous divisions, that the cells pass as a relatively narrow-sized cohort through the different phases of the cell cycle, and that the cells exhibit the expected size and DNA changes as the cells pass through the cell cycle.

The currently accepted view of forcing synchronization needs to be reviewed. The proposal that forcing synchronization works—in contrast to the theoretical and experimental analyses outlined here—requires ongoing scrutiny by those new to the field. There must be a re-evaluation of the current approach to cell-cycle studies, a reanalysis of the results from forced synchronization methods, and a reconsideration of alternate models of the cell cycle (1-6, 10, 18, 22, 27-37).

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