

PERSPECTIVE:

Reanalysis of a proposed protocol for *in vitro* synchronization of mammalian astrocytic cultures by serum deprivation

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ABSTRACT

Serum starvation of astrocytes for a period of time followed by refeeding has been put forward as a method to produce synchronized astrocytes. Here it is proposed that the method neither synchronizes cells nor satisfies basic criteria for cell synchronization. The proposed whole-culture synchronization protocol cannot, in theory, synchronize cells. This is because the cells produced by the proposed serum starvation/refeeding protocol do not reflect the properties of cells of any specific age during the cell cycle. For this reason the proposed protocol will not produce a synchronized culture.

It has been proposed that it is possible to synchronize astrocytes obtained from newborn rat brains by a simple whole-culture treatment of cells.¹ The proposed method was to first starve cells in low serum, and then to re-supplement the cells with serum to obtain a synchronized culture. There are two parts to the detailed protocol: (i) proliferation of astrocytes under optimal conditions *in vitro* until reaching desired confluence; and (ii) synchronization of cultures by serum down-shift and arrest in the G0 phase of the cell cycle.

The key problem with this method is the initial definition of synchronization. The method assumed that “cells arrested in specific phases of the cell cycle [are] synchronized.” I will argue below that producing cells arrested with a particular DNA pattern does not mean that the cells are synchronized. The *sine qua non* of synchronization is that cells grow as a uniform cohort through the cell cycle and then divide synchronously. It is of interest to look at the listed criteria that were applied to the protocol for synchronization of astrocytes¹:

- (a) both normal and tumor cells should be arrested at the same specific cell cycle phase,
- (b) the synchronization must be reversible and non-cytotoxic,
- (c) the metabolic block should target a specific reaction and must be reversible, and
- (d) large quantities of synchronous cells should be obtained

These criteria do not ask whether cells following a particular treatment are synchronized. These criteria do not apply to cell synchronization in any way. Rather, these criteria (taken from the work of Keyomarsi, et al.²) apply to any method that one would want to use to study cells, whether synchronized or not. Thus, a good method should be applicable to different cell lines,

should produce large amounts of cells that are not affected by cytotoxicity, and the treatment should affect a known target. Satisfying these criteria would be an experimental ideal whether the cells were or were not synchronized.

Criteria for “synchronization” have been proposed³, and the method can be tested against these criteria. Some of the criteria (from a longer list) for cell synchronization are:

- 1) If newborn cells are produced by the synchronization method, there should be a minimal increase in cell number for a period of time covering a significant fraction of the interdivision time.
- 2) The rise in cell numbers during division should occur over a relatively small fraction of the total interdivision time. It may be as small as 10% for 90% of the final rise in cell number, or it may be as large as 20-25%. Knowing this value is important in judging a synchronization procedure.
- 3) At the time of synchronous division, the cell number should double. If cell number does not double, that means some cells are dead or altered; this minority of cells could be giving results that obfuscate the results emanating from the majority of dividing cells.
- 4) The DNA distribution of cells should be narrow in the synchronized cells and these distributions should then reflect the movement of cells through the division cycle. Thus, newborn cells should be essentially pure cells with a G1-phase amount of DNA, the DNA content should then move through S-phase contents, there should be a period of time when cells have only G2-phase DNA contents, and then there should be a return to essentially pure G1-phase DNA contents.

- 5) The size distribution of newly synchronized cells should be narrower than the size distribution of the original population, cell size should increase as the cells move through the cell cycle, and during the period of cell division there should be a bi-modal distribution of cell sizes.
- 6) Alignment of cells so that cells all have a particular property in common (e.g., all cells have a G1-phase DNA content) does not mean that the cells are synchronized. Synchronized divisions are the *sine qua non* of synchrony.

The arguments for the proposal that whole-culture treatment cannot synchronize cells and that only selective methods can synchronize cells are given in detail below. Here it will suffice to note that none of these synchronization criteria are satisfied in the original publication for synchronization of astrocytes.¹

An explanation of why cells may be aligned with a particular DNA configuration and yet not be synchronized has been presented before.³⁻¹¹ Here the simple outline of the argument will be presented.

The key point of the analysis is that one can produce a culture with all of the cells having a “G1-phase amount of DNA” but these cells are not equivalent to normal, unperturbed and unsynchronized cells with a G1-phase amount of DNA. Let us say an idealized cell grows from newborn size 1.0 to size 2.0 when the cell divides. Assume, in this example, that DNA synthesis (S phase) starts at size 1.5. Consider two cells, one of size 1.4999 (just at the end of the G1 phase, not yet having started S phase) and the other of size 1.5001 (just started S phase). Now inhibit mass synthesis. The cell of size 1.4999 just sits there, not starting S phase because this cell does not achieve size 1.5; it remains a cell with a G1-phase amount of DNA. The other cell,

at the start of S phase, finishes S phase, completes G2 phase and then divides to produce two newborn cells of sizes 0.75005 (i.e., approximately size 0.75). This is because mass synthesis did not increase from the original size 1.5001 cell as it was proposed that mass increase was inhibited. Are these synchronized cells? No. One cell is of a size that is not like the normal cell, being smaller than the normal smallest cell (the original cells went from size 1.0 to 2.0), and together they are of different sizes (one cell is twice the size of the other). Therefore these two cells do not mimic, as a pair, the DNA content *and* the size distribution of cells at any particular time during the G1 phase.

Upon release of the mass inhibition we can see that the start of DNA replication will occur immediately in the cells of size 1.4999 as they need only 0.0001 units of mass to reach 1.5. In contrast, the cells of size 0.75005 will require approximately one full doubling time to reach size 1.5. Thus there is no synchronous initiation of S phase. By the criteria described above, the cells are not “synchronized.”

For a group of cells to be synchronized it is necessary that they perform a particular cell cycle event (e.g., the start of S phase or the initiation of DNA synthesis) over a period of time that is significantly less than the interdivision time of a culture. If the time to perform a cell-cycle specific event, such as the initiation of S phase, is equal to the interdivision time, then the cells are not synchronized. In the example given here the allegedly synchronized cells do not initiate S phase over a narrow time period, but rather over a time period equal to the doubling time of the culture. Hence the cells are not synchronized. A rigorous analysis of why whole-culture synchronization does not work has been presented for the major classes of whole-culture synchronization.¹¹

Published examples of cells that are proposed to be synchronized but are not synchronized when the actual data are examined are present in the literature.^{2, 9, 12-16}

It is concluded from this analysis that the published protocol for synchronization of astrocytes¹ does not actually synchronize the cells.

Discussion

The interest in the study of events during the cell cycle has led to numerous proposals for methods to synchronize cells. Here one published method is analyzed. It is concluded that the published method neither presented evidence that the cells are synchronized nor considered arguments that the whole-culture method used—serum starvation followed by refeeding serum—does not, and cannot, synchronize cells.

Lest it be thought that it is proposed here that cell synchronization is impossible, it should be noted that it is clearly possible to synchronize cells, but the method must be a selective method. A recently described method, the membrane-elution method (colloquially known as the “baby machine”) has produced well synchronized cultures that do satisfy the criteria for cell synchronization listed here.¹⁷⁻¹⁹

The original protocol for astrocyte synchronization¹ proposed that “Synchronization is particularly important in the study of astrocyte biology, as its application allows astrocytes to re-enter the cell cycle from a state of quiescence (G0), and, under carefully defined experimental conditions, move together into subsequent phases such as the G1 and S phases.” A critique of the G0 concept has been presented and is also applicable to the original protocol proposed for cell synchronization.^{6, 10, 20}

The critique of the proposed synchronization protocol presented here is important for two reasons. First, if the method for synchronizing astrocytes does not actually work, it is important to point this fact out to the workers in the field before the method is used to study the cell cycle. Without a critical analysis of the original method much effort and experimental work might be expended on a method that does not work. Second, while it is very likely the case that numerous methods have been proposed to synchronize cells that use these whole-culture methods, it is not well-recognized that, in theory, such methods do not work.

References

1. Chou RC, Langan TJ. In vitro synchronization of mammalian astrocytic cultures by serum deprivation. *Brain Res Brain Res Protoc.* Jul 2003;11(3):162-7.
2. Keyomarsi K, Sandoval L, Band V, Pardee AB. Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. *Cancer Res.* Jul 1 1991;51(13):3602-9.
3. Cooper S, Shedden K. Microarray analysis of gene expression during the cell cycle. *Cell & Chromosome.* 2003;2:1.
4. Cooper S. G1 and S phase gene expression cannot be analyzed in mammalian cells synchronized by inhibition. *Microb Comp Genomics.* 1997;2(4):269-73.
5. Cooper S. Mammalian cells are not synchronized in G1-phase by starvation or inhibition: considerations of the fundamental concept of G1-phase synchronization. *Cell Prolif.* 1998;31(1):9-16.
6. Cooper S. On the proposal of a G0 phase and the restriction point. *FASEB J.* Mar 1998;12(3):367-73.
7. Cooper S. The continuum model and G1-control of the mammalian cell cycle. *Prog Cell Cycle Res.* 2000;4:27-39.
8. Cooper S, Shayman JA. Revisiting retinoblastoma protein phosphorylation during the mammalian cell cycle. *Cell Mol Life Sci.* Apr 2001;58(4):580-95.
9. Cooper S. Reappraisal of G1-phase arrest and synchronization by lovastatin. *Cell Biol Int.* 2002;26:715-27.
10. Cooper S. Reappraisal of serum starvation, the restriction point, G0, and G1-phase arrest points. *FASEB J.* 2003;17:333-40.
11. Cooper S. Rethinking synchronization of mammalian cells for cell-cycle analysis. *Cell Mol Life Sci.* 2003;6:1099-106.
12. Di Matteo G, Fuschi P, Zerfass K, et al. Transcriptional control of the Htf9-A/RanBP-1 gene during the cell cycle. *Cell Growth Differ.* Oct 1995;6(10):1213-24.
13. Shedden K, Cooper S. Analysis of cell-cycle-specific gene expression in human cells as determined by microarrays and double-thymidine block synchronization. *Proc Natl Acad Sci USA.* Apr 2 2002;99(7):4379-84.

14. Whitfield M, Sherlock G, Saldanha A, et al. Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Molecular Biology of the Cell*. 2002;13:1977-2000.
15. Cho RJ, Campbell MJ, Winzeler EA, et al. A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol Cell*. Jul 1998;2(1):65-73.
16. van der Meijden CM, Lapointe DS, Luong MX, et al. Gene profiling of cell cycle progression through S-phase reveals sequential expression of genes required for DNA replication and nucleosome assembly. *Cancer Res*. Jun 1 2002;62(11):3233-43.
17. Thornton M, Eward KL, Helmstetter CE. Production of minimally disturbed synchronous cultures of hematopoietic cells. *Biotechniques*. May 2002;32(5):1098-105.
18. Helmstetter CE, Thornton M, Romero A, Eward KL. Synchrony in human, mouse, and bacterial cell cultures. *Cell Cycle*. 2003;2:42-5.
19. Cooper S. Minimally disturbed, multi-cycle, and reproducible synchrony using a eukaryotic "baby machine" *Bioessays*. 2002;24:499-501.
20. Cooper S. On G0 and cell cycle controls. *Bioessays*. Nov 1987;7(5):220-3.