On the Persistence of Whole-Culture Synchronization for Cell-Cycle Analysis

Stephen Cooper

Department of Microbiology and Immunology

University of Michigan Medical School

Ann Arbor, Michigan 48109-0620

E-mail: *cooper@umich.edu*

Running Title: Persistence of Whole-Culture Synchronization

Corresponding Author: Stephen Cooper, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-0620;

E-mail: *cooper@umich.edu*; Phone: 734-764-4215, FAX: 734-764-3562

Total character count: 25,019 (total, with spaces); no figures; 19 pages submitted,

56 words in Abstract

Abstract

Whole-culture methods of eukaryotic cell synchronization are the most widely used approaches to cell-cycle analysis. Despite this wide-spread use, theoretical considerations and experimental evidence indicate that whole-culture methods cannot synchronize cells. Why, despite these clear experimental and theoretical critiques, are these methods still so widely used? Reasons for the persistence of whole-culture synchronization methods are explored.

It is generally believed that it is possible to synchronize cells by whole-culture treatment of growing cells. “Whole-culture synchronization” means that the cells in a previously unsynchronised culture are induced to form a synchronized culture by applying a common treatment to all cells. When cells of various cell-cycle ages are all treated identically and growth arrested, it is generally presumed that the cells arrest at a common cell-cycle age. It is further believed that upon release from growth arrest these cells can generate a synchronized culture. One common and often-described synchronization method involves placing growing mammalian cells in a low-serum medium producing growth arrest. The arrested cells are assumed to enter a G0 or a G0/G1 phase, or to arrest at a 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. Treatment of a growing culture with the cholesterol-lowering drug lovastatin has even 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”. Raising the temperature of yeast cells with specific temperature-sensitive defects has also been proposed as a synchronization method.

The 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.

It is not the purpose of this discussion to review or prove that whole-culture synchronization cannot work in theory or in practice. That has been done in other published papers as discussed briefly below. The main purpose of this analysis is to try to understand some of the reasons why, despite evidence that these methods do not work, the whole-culture approach has remained so dominant an approach to cell-cycle analysis.

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

Despite this consensus around whole-culture synchronization methodology, it is theoretically impossible to synchronize cells by any whole-culture method or treatment[1-3]. This assertion, unsupportive of the current and widely used whole-culture

synchronization methodologies, is generally and widely ignored. The field continues to use methods that, in theory and in practice, do not synchronize cells. It is not that synchronization of eukaryotic or mammalian cells is impossible. A discussion of successful *selective* synchronization is presented below. What is impossible is cell synchronization using non-selective, whole-culture treatments where all cells are proposed to be aligned at some point in the cell cycle by a common treatment of all cells.

Again, it is not the purpose of this paper to prove or even review the evidence that whole-culture synchronization does not work; this has been done in numerous published papers. I take the published critiques of whole-culture synchronization as valid; none of these critiques have ever been refuted. Nor is it the purpose of this paper to present new evidence that whole-culture synchronization does not work. The experimental evidence critical of whole-culture methods has been described in numerous publications. Rather, it is the purpose of this paper to explore *why* these ideas and experiments critiquing whole-culture synchronization have not eliminated these whole-culture, non-selective, synchronization methods from the canon of cell biology methods.

Because the ideas and experiments critical of whole-culture synchronization methods are not well known, it is nonetheless necessary to briefly restate the theoretical and experimental arguments against whole-culture synchronization before discussing why these whole-culture methods persist in the laboratory and the literature, The critique of whole-culture methods are based on both a theoretical analysis as well as critical experimental work demonstrating that whole-culture synchronization methods do not

work. It is for these reasons that the use of whole-culture methods must be reassessed. If the whole-culture methods proposed to synchronize cells do not actually synchronize cells, then any results regarding cell-cycle control that are derived from these experiments must be re-examined. Given that whole-culture methods of synchronization do not work (discussed below), the question addressed in this paper is simply "Why do whole-culture methods continue to dominate the approach to cell-cycle studies?"

The conceptual argument against whole-culture 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 cells at the end of the cell cycle that are dividing. Cells of intermediate cell-cycle ages have intermediate sizes. In order to produce a synchronized culture, one must align cells with uniform DNA contents. But having uniform DNA contents is not sufficient for synchronization. In addition to a uniform cellular DNA content, 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. Thus, if cells grow through the division cycle from size 1.0 to 2.0, the starting cells of a synchronized culture that have a G1-phase amount of DNA should have, for example, cells of size 1.1 with some variance due to statistical and experimental variation. If the initial cells after whole-culture synchronization retained the two-fold variation in cell sizes, then even if cells had a uniform DNA content it is not possible to ascribe to these cells their identity or similarity to cells of a particular cell age during the normal, unperturbed cell cycle.

For the simple case of arrest with a G1-phase amount of DNA, the argument points out that cessation of cell cytoplasm increase leads to no further initiations of S phases. But cells in S- and G2/M-phases do complete these phases, and go on to divide, producing cells with a G1-phase amount of DNA. These cells, along with the original cells remaining with a G1-phase amount of DNA (due to the absence of initiation of S phases) have a uniform DNA content. But the mass distribution is not narrowed, and remains as broad as before[1]. Thus, the cells may have a G1-phase amount of DNA, but are not synchronized, do not produce a synchronized culture, and the cells as a group are not representative of any particular cell age during the normal cell cycle.

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 using these whole-culture methods 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 stop 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 whole-culture 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 whole-culture methods have been strongly supported by much experimental evidence. A reanalysis of the whole-culture 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 clearly not synchronized. A direct demonstration of the lack of synchronization of cells by a whole-culture 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] indicated that the original data on synchronization[11] was consistent with a lack of synchrony. Furthermore, data showing that lovastatin-treated cells are arrested in the G1-phase of the cell cycle[12,13] has been reinterpreted; the cells were not actually arrested in any particular phase of the cell cycle[10]. Other laboratories have also presented data that indicate no synchronization with 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 whole-culture 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 whole-culture 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 the clear theoretical and experimental support for the proposal that whole-culture methods do not synchronize cells, does the use of these whole-culture synchronization methods still hold sway over the field of cell biology and cell-cycle studies?

One answer to this question points to the absence of explicit criteria to define a synchronized culture. The ease of fluorescence assisted cell sorting (FACS) 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 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 published DNA analyses during cell growth are presented to support a presumed synchronization experiment, the actual support for synchronization is not there. And sometimes the data strongly 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. To demonstrate synchronization, the data must show that cells

divide over a relatively short span of time (i.e., relative to the total interdivision time), and points between these rises in cell numbers should show clear plateaus of non-division. Cell division analysis of whole-culture 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 appears to eliminate 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—more technically referred to as membrane-elution—produces, by selection, 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 in the initial cells and during synchronized growth[25-27], satisfying the basic and stringent criteria for synchronized growth. If cells produced by whole-culture synchronization are truly synchronized, then one should be able to see synchronized divisions. Rather than considering the absence of division synchrony as an indication of the variability of eukaryotic cell interdivision times, the absence of division synchrony is actually strong 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. The cell numbers in the synchronized cultures eluted from the membrane follow a synchronized pattern without any connecting lines to emphasize synchrony. Furthermore, 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 whole-culture 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 and widely described whole-culture methods. This uncritical use of whole-culture methods may be due to the simple fact that researchers haven't the time to re-examine the original experiments and whole-culture 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].

The ubiquitous reporting of whole-culture synchronization without strict criteria to demonstrate synchronization is an example of "confirmation bias", where a researcher tends to accept results as being consistent with previously reported results rather than proposing that a particular method does not work. If one does not "confirm" a result that is widely accepted, it may be a source of discomfort and embarrassment that the researcher cannot repeat a particular method. This is one source of such "confirmation bias".

Another reason these whole-culture 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 generated the idea that it is easy to synchronize cells by simple whole-culture treatments. But merely aligning cells with a common DNA content (e.g., assuming "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

synchronizing cells by arresting them in G0 phase." It is more difficult 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 replenishing the serum and taking samples every hour, although I have yet to show that the cells are truly synchronized and have synchronized divisions."

New researchers entering the field see descriptions of whole-culture methods in textbooks and assume that these methods describe a reality that can be used to synchronize cells. It is rare that a researcher goes back to relatively ancient work (e.g., 29 years old) and checks to see whether the experiments have an alternative explanation.

The extremely large number of papers using whole-culture methods is a fundamental feature explaining the persistence of whole-culture synchronization. The response to criticism of whole-culture synchronization is usually in the form of a question: "How can a few experiments, and a few theoretical arguments, criticizing whole-culture synchronization methods compare with the hundreds and probably thousands of papers reporting that such whole-culture methods do synchronize cells?"

A rigorous adherence to scientific principles requires that those who wish to use whole-culture methods respond to the theoretical and experimental critiques raised here. Arguments based on the number of papers using whole-culture synchronization do not answer the concerns raised here regarding these methods. The validity of whole-culture synchronization methods should not be determined by majority vote. Experiments using

whole-culture 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. More than one cell cycle should also be apparent in a good synchronization experiment.

The continuous promotion of whole-culture methods of synchronization in numerous papers and reviews, written by leaders in the field of cell biology and cell-cycle research is possibly the primary reason for the continued use of these ineffectual methods. There is a “will to believe” that arises from the enormity of the published literature using these whole-culture methods that makes criticism of these methods difficult to accept. It is the purpose of this article to advocate that whole-culture methods should be subjected to a critical analysis and not be accepted merely because they have become the norm of cell-cycle research.

The widespread acceptance of whole-culture synchronization needs to be reviewed. The proposal that whole-culture synchronization works—in contrast to the theoretical and experimental analyses outlined here—requires ongoing scrutiny by those new to the field, as well as those with long-term tenure in cell-cycle studies. There must be a re-evaluation of the current approach to cell-cycle studies, a reanalysis of the results from whole-culture synchronization methods, and a reconsideration of alternate models of the cell cycle[1-6,10,18,22,27-37].

Acknowledgements: A number of individuals have read and commented on this article, but key comments leading to critical improvements were made by Katherine Spindler, Michele Swanson, Marc R. Roussel, Victor Norris, Nanne Nanninga, Richard D'Ari, and Frederick Neidhardt. Alexandra Cooper was an invaluable aide in editing this paper, and giving it the proper tone.

Correspondence should be addressed to cooper@umich.edu. Additional material on this subject can be viewed at www.umich.edu/~cooper.

References

1 Cooper, S. (2003) Rethinking Synchronization of mammalian cells for cell-cycle analysis. *Cell Mol Life Sci* 6, 1099-1106

2 Cooper, S. (1998) Mammalian cells are not synchronized in G1-phase by starvation or inhibition: considerations of the fundamental concept of G1-phase synchronization. *Cell Prolif* 31 (1), 9-16

3 Cooper, S. (2000) The continuum model and G1-control of the mammalian cell cycle. *Prog Cell Cycle Res* 4, 27-39

4 Cooper, S. (1997) G1 and S phase gene expression cannot be analyzed in mammalian cells synchronized by inhibition. *Microb Comp Genomics* 2 (4), 269-273

5 Cooper, S. (1987) On G0 and cell cycle controls. *Bioessays* 7 (5), 220-223

6 Cooper, S. (1998) On the proposal of a G0 phase and the restriction point. *FASEB J* 12 (3), 367-373

7 Di Matteo, G. et al. (1995) Transcriptional control of the Htf9-A/RanBP-1 gene during the cell cycle. *Cell Growth Differ* 6 (10), 1213-1224

8 Shedden, K. and Cooper, S. (2002) Analysis of cell-cycle-specific gene expression in human cells as determined by microarrays and double-thymidine block synchronization. *Proc Natl Acad Sci USA* 99 (7), 4379-4384

9 Cho, R.J. et al. (2001) Transcriptional regulation and function during the human cell cycle. *Nat Genet* 27 (1), 48-54

10 Cooper, S. (2002) Reappraisal of G1-phase arrest and synchronization by lovastatin. *Cell Biol Int* 26, 715-727

11 Keyomarsi, K. et al. (1991) Synchronization of tumor and normal cells from G1 to multiple cell cycles by lovastatin. *Cancer Res* 51 (13), 3602-3609

12 Rao, S. et al. (1998) Lovastatin mediated G1 arrest in normal and tumor breast cells is through inhibition of CDK2 activity and redistribution of p21 and p27, independent of p53. *Oncogene* 17 (18), 2393-2402

13 Rao, S. et al. (1999) Lovastatin-mediated G1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. *Proc Natl Acad Sci U S A* 96 (14), 7797-7802

14 Barrett, K.L. et al. (2002) Cyclin b1 promoter activity and functional cdk1 complex formation in G1 phase of human breast cancer cells. *Cell Biol Int* 26 (1), 19-28

15 Zetterberg, A. and Larsson, O. (1991) Coordination between cell growth and cell cycle transit in animal cells. *Cold Spring Harb Symp Quant Biol* 56, 137-147

16 Zetterberg, A. and Larsson, O. (1985) Kinetic analysis of regulatory events in G1 leading to proliferation or quiescence of Swiss 3T3 cells. *Proc Natl Acad Sci U S A* 82 (16), 5365-5369

17 Zetterberg, A. et al. (1995) What is the restriction point? *Curr Opin Cell Biol* 7 (6), 835-842

18 Cooper, S. (1981) The continuum model: application to G1-arrest and G(O). In *Cell Growth* (Nicolini, C., ed.), pp. 315-336, Plenum Press

19 Urbani, L. et al. (1995) Dissociation of nuclear and cytoplasmic cell cycle progression by drugs employed in cell synchronization. *Exp Cell Res* 219 (1), 159-168

20 Pardee, A.B. (1989) G1 events and regulation of cell proliferation. *Science* 246 (4930), 603-608

21 Pardee, A.B. (1974) A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci U S A* 71 (4), 1286-1290

22 Cooper, S. (2003) Reappraisal of Serum Starvation, the Restriction Point, G0, and G1-phase Arrest Points. *FASEB J* 17, 333-340

23 van der Meijden, C.M. et al. (2002) Gene profiling of cell cycle progression through S-phase reveals sequential expression of genes required for DNA replication and nucleosome assembly. *Cancer Res* 62 (11), 3233-3243

24 Whitfield, M. et al. (2002) Identification of Genes periodically expressed in the human cell cycle and their expression in tumors. *Molecular Biology of the Cell* 13, 1977-2000

25 Thornton, M. et al. (2002) Production of minimally disturbed synchronous cultures of hematopoietic cells. *Biotechniques* 32 (5), 1098-1105

26 Helmstetter, C.E. et al. (2003) Synchrony in Human, Mouse, and Bacterial Cell Cultures. *Cell Cycle* 2, 42-45

27 Cooper, S. (2002) Minimally Disturbed, Multi-Cycle, and Reproducible Synchrony using a Eukaryotic "Baby Machine" *Bioessays* 24, 499-501

28 Cooper, S. (1979) A unifying model for the G1 period in prokaryotes and eukaryotes. *Nature* 280, 17-19

29 Cooper, S. (1982) The continuum model: statistical implications. *J Theor Biol* 94 (4), 783-800

30 Cooper, S. (1988) The continuum model and c-myc synthesis during the division cycle. *J Theor Biol* 135 (3), 393-400

31 Cooper, S. (1991) *Bacterial Growth and Division*, Academic Press

32 Cooper, S. (1998) On the interpretation of the shortening of the G1-phase by overexpression of cyclins in mammalian cells. *Exp Cell Res* 238 (1), 110-115

33 Cooper, S. et al. (1999) Phosphorylation-dephosphorylation of retinoblastoma protein not necessary for passage through the mammalian cell division cycle. *IUBMB Life* 48 (2), 225-230

34 Cooper, S. (2000) Toward a standard system for the mammalian cell cycle. *ASM News* 66, 71-75

35 Cooper, S. (2001) Revisiting the relationship of the mammalian G1 phase to cell differentiation. *J Theor Biol* 208 (4), 399-402

36 Cooper, S. and Shayman, J.A. (2001) Revisiting retinoblastoma protein phosphorylation during the mammalian cell cycle. *Cell Mol Life Sci* 58 (4), 580-595

37 Cooper, S. (2002) The Schaechter-Bentzon-Maaløe experiment and the analysis of cell cycle events in eukaryotic cells. *Trends in Microbiology* 10, 169-173