



# Is whole-culture synchronization biology's 'perpetual-motion machine'?

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**Whole-culture or batch synchronization cannot, in theory, produce a synchronized culture because it violates a fundamental law that proposes that no batch treatment can alter the cell-age order of a culture. In analogy with the history of perpetual-motion machines, it is suggested that the study of these whole-culture 'synchronization' methods might lead to an understanding of general biological principles even though these methods cannot be used to study the normal cell cycle.**

Three points can be made regarding perpetual-motion machines: first, proposals of this nature keep appearing and will continue to appear no matter how many previous attempts have failed. Second, such machines violate fundamental physical laws, and we accept that a machine violating these laws cannot be built. Third, the analysis of these machines might still be of interest, if only as a way of understanding how to apply the fundamental laws of thermodynamics to real situations. As discussed here, the same list can be applied to whole-culture synchronization methods.

## The lure and persistence of perpetual-motion machines

For centuries researchers have attempted to develop or invent the Holy Grail of energy production – a perpetual-motion machine. Recorded attempts to achieve perpetual motion date back at least 15 centuries. Many simply think that it must be possible, and that if they only try hard enough, they will find a solution. Up until 200 years ago, this belief could be forgiven. Even Leonardo da Vinci hoped to develop such an instrument. But with our understanding of the nature of energy, and the first and second laws of thermodynamics, we can only conclude that recent attempts at perpetual-motion machines are surely deluded and doomed to failure.

Attempts to develop these instruments have ranged from ludicrous to intelligent in design. There have been attempts such as a self-blowing windmill, a perpetual ball-moving machine, self-powered water mills, a machine running on capillary action, a permanent magnet-motor machine, a self-acting pump, and recently a cold fusion, laser-activated magnetic motor. As recently as January 2000 someone proposed to have developed a 'self-running engine.'

Through modern physics we now understand why attempts at perpetual-motion machines cannot succeed. Perpetual motion machines violate a fundamental law of physics that says that energy cannot be created from nothing. If we were given the plan for a perpetual-motion

machine we would not hesitate to throw it in the trash, although we might not immediately know precisely what was wrong with the proposal.

Nevertheless, although the possibility of constructing a perpetual-motion machine is dead as a topic of serious discussion, the analysis of proposed perpetual-motion machines is often interesting. The study of such attempts can expose conceptual errors and illustrate important theoretical points regarding the laws of thermodynamics.

## Whole-culture synchronization and the law of conservation of cell-age order

Whole-culture synchronizing methods are those in which all of the cells in a culture are treated identically to produce a set of cells that are therefore presumed to be of the same cell-cycle age; that is, the cells are forced to be of the same age and are thus 'synchronized'. Numerous methods have been proposed for the 'synchronization' of a population of cells by treating a cell culture to arrest cells at a particular point in the cell cycle. Releasing the arrested cells is then proposed to lead to synchronous growth. For example, one of the most popular methods for synchronizing animal cells is to starve cells in low serum, and then add back serum at a later time. This approach has been used in innumerable papers to produce a synchronized culture.

These synchronization attempts, although widely accepted, are as doomed to failure as perpetual-motion machines. This is because whole-culture synchronization methods violate a fundamental law of cell growth, the law of conservation of cell-age order (LCCAO). This law proposes that, in theory, attempts at batch synchronization cannot work. However, it is of interest to study some of these proposed and published (and even widely accepted) methods to understand some basic principles of cell biology and the cell cycle.

What is the LCCAO? As originally written the law states:

'For any genetically pure bacterial (or eukaryotic cell) culture growing in balanced exponential growth, and ignoring statistical variation, there is no batch treatment of the culture that can lead to an alteration of the cell-age order [1].'

A corollary of this law is that no batch treatment of an exponentially growing culture can produce a synchronized culture.

Although this law was originally written for bacteria, in which case it is clearly understood why such whole-culture methods do not work [1], the law is directly applicable to

eukaryotic cells. It is in the eukaryotic context that we shall discuss whole-culture (or batch) synchronization.

A distinction must be made between selection methods and batch or whole-culture methods. Selection methods, in which a portion of the growing population is selected from the total population can, in theory, and even in practice, produce a synchronized culture. The recent development of a eukaryotic 'baby machine' has produced a synchronized culture that gives at least three synchronized divisions [2–4]. The 'baby-machine' method produces a synchronized culture by binding cells to a membrane, allowing cells to grow on the membrane, and having only newborn cells released from the membrane at the moment of division. Membrane-elution is a classic selection method because only newborn cells are selected from the population to produce the synchronized culture. Batch or whole-culture methods are those in which there is no selection. In a whole-culture method all of the cells are forced, Procrustean-like, into a common state to produce the synchronized culture. Let us examine why, in theory, such whole-culture methods do not work.

Cells growing in exponential culture are of different ages between newborn cell age (age 0.0) and dividing cell age (age 1.0). During exponential growth, considering the population of cells at a particular instant, cells closer to division will divide before cells further from division. Also, cells closer to division are older (i.e. further in time from when they were born by division of a mother cell) than the younger cells that are further from division. The LCCAO states that one cannot treat a batch of such exponentially growing cells to cause cells that are further from division at the time of treatment to divide before – or at the same time as – other cells that were scheduled to divide earlier or at a different time than the selected cells before treatment. The corollary of the LCCAO is that one cannot treat a culture to adjust the cell ages so cells with disparate ages now come together having the same cell-cycle age.

#### Why whole-culture synchronization does not work

The simplest example of this law in application is in the case of cell-growth arrest such that after treatment all cells have G1-phase amount of DNA. [5–11]. Consider that in the exponentially growing culture the cells range between sizes 1.0 (newborn size) and 2.0 (dividing cell size). Now inhibit cell mass increase so that all cells remain fixed in their cell mass. That is, the mass of a cell remains fixed for the entire treatment period. Cells that have not reached the size for initiation of S phase (e.g. cells of size 1.6) remain cells with a G1-phase amount of DNA. Cells that are above size 1.6, and are in either S- or G2/M-phases complete these phases, divide and produce daughter cells with a G1-phase amount of DNA. A cell that just started S phase, which could be said to be of size 1.60001, completes S- and G2/M-phases, dividing to produce two daughter cells of size  $\sim 0.8$ . This is because the treatment of the cell inhibited cytoplasmic mass increase during the time that the cells completed S- and G2/M-phases. Observe that the largest cell from the G1-phase population (the cell just at the end of G1-phase) has a size of 1.5999, just below size 1.6, the size at which cells would initiate DNA replication, while the progeny of the very early S-phase

cell are now approximately size 0.8. The spread in cell sizes after all cells capable of dividing have divided goes from  $\sim 0.8$  to  $\sim 1.6$ . The size distribution after the inhibition is therefore just as wide – cells have sizes that spread over a twofold range – as in the original population. There has been no narrowing of the size distribution of cells. All of these cells have a G1-phase amount of DNA. But it is clear that one should not say that these cells are 'in G1-phase'. Or 'arrested at a point in G1-phase'.

This analysis can be generalized to say that, in a synchronized culture, cells must have not only the DNA content but also the size characteristics of cells of the particular age at the point in the division cycle of cells in an unperturbed culture. In the example of inhibition of mass increase (above), at the end of the inhibition period the cells varied in size over a factor of two (size 0.8 to 1.6) and some cells (e.g. size 0.8) were not present at a size equivalent to any cell size in the normal division cycle size range (1.0–2.0).

Mass arrest, as well as other methods, such as arrest at mitosis or arrest by inhibition of DNA synthesis have been analyzed in detail [5].

It might be of value to put cells in a state that is uniform with respect to a particular chemical or physical parameter (irrespective of synchronization) to elicit, for example, a uniform response to some inhibiting condition. Thus, if one wanted cells going through mitosis as a group, or cells arrested in a mitotic state, use of a mitotic inhibitor might be satisfactory. If one wanted to perform an *in vitro* fertilization on a cell with a G1-phase amount of DNA, it might be satisfactory to arrest cells 'with a G1-phase amount of DNA'. However, is not satisfactory to call cells released from mitotic inhibition, or cells with a G1-phase amount of DNA, or cells in any other batch or whole-culture arrest condition, 'synchronized'. They might be aligned at or for a particular event, or have a particular cell composition, but they are not synchronized.

There is experimental support for these theoretical proposals. Experiments using a double-thymidine block (i.e. two treatments with excess thymidine) were proposed to synchronize human cells [12] but a detailed analysis of the data indicated that the cells were not synchronized [13]. This was supported by an analysis that showed that the second peak of gene expression could be equally above or below the height of the first peak of gene expression; this is not expected if the cells are truly synchronized [13]. Examination of other published data presented to support synchronization by whole-culture methods in fact supports the proposal that such cells are not synchronized [10,14–17].

#### Selective methods can synchronize cells

Of course, these strictures regarding synchronization do not apply to selective procedures in which cells are not all treated identically. A subset of cells of a certain age selected from the original asynchronous population could constitute, in theory, a synchronized culture. Two well-known selective methods for producing synchronized cultures are elutriation (hydrodynamic separation in a centrifuge) or mitotic selection (selection of cells released from a substrate during mitosis). Although these methods

work in theory, in practice they do not give sharp and well-defined synchronous patterns of cell growth and division. For elutriation (i.e. hydrodynamic separation of cells according to the sedimentation coefficient of individual cells) it is likely that both size and shape affect the passage of cells through a gradient. Cells of different sizes might be together in a fraction because cell shape made the cells travel together, rather than because of their common age. Although mitotic selection might work in theory, in practice it has not been used extensively.

### The development of the eukaryotic 'baby machine'

There have been many arguments raised against the proposal that forced synchronization methods are unable to produce a synchronized culture. Some have said that perhaps methods are not very 'efficient' at synchronizing cells, but that some other whole-culture method does it better. Or some have said that one should not expect good synchronization of cell division because stochastic variation in interdivision times would lead to a rapid decay of cell division synchrony. All of these arguments pale into insignificance when confronted with a brilliant new development in cell synchronization, the eukaryotic 'baby machine'.

The eukaryotic 'baby machine' is a method, developed in the laboratory of Dr Charles Helmstetter, that produces newborn cells from cells growing on a membrane [2–4]. These cells give four synchronized growth cycles separated by three clear synchronous divisions. The cells are unperturbed, give the expected size and DNA distributions after elution from the baby machine and show clear and unambiguous synchronized growth. Synchrony of eukaryotic cells is not impossible, but it is impossible using batch or forcing procedures. Most important, regarding the baby-machine analysis presented here, we now have a 'gold standard' against which other synchronization methods should be measured. (An excellent description

of the method and result obtained can be read directly at [hyper.fit.edu/biology/Files/Helmstetter.pdf](http://hyper.fit.edu/biology/Files/Helmstetter.pdf).) If one cannot demonstrate that a particular method gives reproducible, unperturbed, normal, sequential, synchronized cell cycles, now one cannot brush off this result by saying that the synchrony is there but the synchrony is not that good. The alternative explanation that the cells were never synchronized in the first place is now a valid interpretation of the absence of synchronized divisions following whole-culture synchronization experiments.

### Whole-culture synchronization and perpetual motion

We can now see the analogy of whole-culture synchronization and perpetual-motion machines. First, both have been with us a long time and it appears that they will be with us in the future – although both projects are doomed to failure. Second, both projects are doomed because they violate fundamental laws.

The final part of the analogy with perpetual-motion machines is to study these synchrony methods to glean from them some basic laws of biology. The main idea to be taken from the failure of cells arrested with a G1-phase amount of DNA to produce a synchronized culture is that there is no need to postulate G1-phase arrest points or restriction points [5,6,8–10,14]. Cells achieve a uniform DNA content with a G1-phase amount of DNA because of two processes. Inhibition of mass increase (e.g. by serum starvation or amino acid starvation) leads to cessation of initiations of S-phase, and S- and G2-phases in progress are completed even though mass increase ceases. These two processes lead to a population of cells all with a G1-phase amount of DNA. But these cells are of different sizes and do not produce a synchronized culture. The arrest with a G1-phase amount of DNA (not 'arrest at a point in G1-phase') was observed without the postulation of any specific G1-phase function at which cells are arrested.

#### Box 1. Criteria for successful synchronization of cells

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 might be as small as 10% for 90% of the final rise in cell number, or 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 it 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) There should be at least two successive cycles available for analysis. If only one cycle is analyzed, the results might merely reflect artifacts or perturbations resulting from synchronization. Presumably, but not necessarily, these artifacts would be eliminated in the second cycle.

5) Successive generations (i.e. the time between rises in cell number) should be of equal length and equal to the doubling time of cells in exponential growth.

6) Data points should show synchrony without any need to connect points or draw a suggestive line indicating synchrony. The data should speak for itself.

7) 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.

8) 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.

9) Cell numbers should be determined by a method that eliminates investigator bias. For example, electronic cell counting is to be preferred to microscope counting chambers.

10) Only selection methods can give synchrony. Whole-culture methods, using inhibition or starvation, cannot synchronize cells. This is not so much a criterion, as a theoretical rule regarding synchronization in general.

11) 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 main distinguishing characteristic of a synchronized culture is that the cells divide synchronously. But there is more to a well-synchronized culture, and several criteria for recognizing a synchronized culture have been presented [11] and are summarized in Box 1 (also see <http://www.cellandchromosome.com/content/2/1/1>).

In time, it is hoped that these ideas will spread through the field of cell biology and cell cycle studies and that we might see a diminution of the use of whole-culture synchronization procedures. Until then, the results from such synchronization methods could be of interest as responses to the synchronizing procedure, but these results cannot, and should not, be used to make deductions about the normal cell cycle.

So although perpetual-motion machines will always find their champions, and whole-culture synchrony theirs, we can only look upon these attempts with a wry sadness and hope that at a minimum they do not deleteriously affect our understanding of thermodynamics or of the cell cycle.

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#### References

- 1 Cooper, S. (1991) *Bacterial Growth and Division*, Academic Press
- 2 Helmstetter, C.E. *et al.* (2003) Synchrony in human, mouse, and bacterial cell cultures. *Cell Cycle* 2, 42–45
- 3 Thornton, M. *et al.* (2002) Production of minimally disturbed synchronous cultures of hematopoietic cells. *Biotechniques* 32, 1098–1105
- 4 Cooper, S. (2002) Minimally disturbed, multi-cycle, and reproducible synchrony using a eukaryotic “baby machine”. *Bioessays* 24, 499–501
- 5 Cooper, S. (2003) Rethinking synchronization of mammalian cells for cell-cycle analysis. *Cell. Mol. Life Sci.* 60, 1099–1106
- 6 Cooper, S. (2003) Reappraisal of serum starvation, the restriction point, G0, and G1-phase arrest points. *FASEB J.* 17, 333–340
- 7 Cooper, S. (2002) The Schaechter-Bentzon-Maaløe experiment and the analysis of cell cycle events in eukaryotic cells. *Trends Microbiol.* 10, 169–173
- 8 Cooper, S. and Shayman, J.A. (2001) Revisiting retinoblastoma protein phosphorylation during the mammalian cell cycle. *Cell. Mol. Life Sci.* 58, 580–595
- 9 Cooper, S. (2000) The continuum model and G1-control of the mammalian cell cycle. *Prog. Cell Cycle Res.* 4, 27–39
- 10 Cooper, S. (2002) Reappraisal of G1-phase arrest and synchronization by lovastatin. *Cell Biol. Int.* 26, 715–727
- 11 Cooper, S. and Shedden, K. (2003) Microarray analysis of gene expression during the cell cycle. *Cell Chromosome* 2, 1
- 12 Cho, R.J. *et al.* (2001) Transcriptional regulation and function during the human cell cycle. *Nat. Genet.* 27, 48–54
- 13 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. U. S. A.* 99, 4379–4384
- 14 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, 9–16
- 15 Di Matteo, G. *et al.* (1995) Transcriptional control of the Htf9-A/RanBP-1 gene during the cell cycle. *Cell Growth Differ.* 6, 1213–1224
- 16 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, 3233–3243
- 17 Whitfield, M. *et al.* (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Mol. Biol. Cell* 13, 1977–2000