Minimally disturbed, multicycle, and reproducible synchrony using a eukaryotic "baby machine"

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Summary
A eukaryotic ''baby machine'' has been developed that produces synchronized cultures that display up to four synchronous cell cycles.[1] That such cells can be produced implies that methods unable to produce successive synchronized cell cycles may not actually synchronize cells. But most important, the baby machine method now opens the way for the study of the cell cycle of minimally disturbed, artifact-free, well-synchronized, mammalian cells. *BioEssays* 24:499–501, 2002.

Introduction

The ''baby machine'' is a method or apparatus for producing large amounts of minimally disturbed, normal, synchronized cells. For over 30 years, a bacterial baby machine has been fundamental to the study and analysis the bacterial division cycle.[2] Using the bacterial baby machine, the patterns of DNA replication, cell wall synthesis, plasmid replication, protein synthesis, and membrane synthesis during the division cycle have been determined, leading to a clear and coherent description of the bacterial division cycle. The same methodology can now be applied to the analysis of mammalian cells with the development of a mammalian baby machine.[1] Most important, as explained below, the very existence of the baby machine methodology has implications for our understanding of the eukaryotic cell cycle. It is not often that the mere development of a method has important biological meaning just because the method works. But as discussed below, this is just the case.

The baby machine or membrane-elution method is extremely simple. Growing cells are gently filtered onto a nitrocellulose membrane that has been treated to allow cells to bind. The filter is inverted, warm medium is pumped through the membrane, and the bound cells grow on the membrane. At cell division, one daughter cell remains attached to the membrane while the other daughter cell is eluted with the medium. Eluted cells collected for a short period of time produce a synchronized culture with the cells dividing synchronously and passing as a cohort through the sequential stages of the cell cycle.

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The bacterial "baby machine"

Charles Helmstetter working as a post-doc at the NIH developed the bacterial membrane-elution method in the early 1960s.[3,4] A few years earlier, Maruyama and Yanagita[5] had proposed a method for synchronizing cells where bacteria were sucked through a large pile of filter papers. The concept was that the smaller cells would percolate preferentially through the filter papers. The first cells eluted would thus be the smallest, and the youngest, cells from the culture. These cells would then grow as a synchronized culture. Because the cells were washed through the filter paper with warm medium and no starvation or inhibition was used, it was believed that these cells would be synchronized without perturbations or artifacts. Helmstetter worked at synchronizing bacteria using the multilayer filter paper technique.

The availability, at this time, of the newly developed electronic particle counter, the Coulter Counter, allowed Helmstetter to accurately measure cell number and cell size without resorting to plate counts or a microscope counting chamber. These accurate measurements showed that the cells eluted from the filter papers were only minimally selected for small cells and these cells did not produce synchronized divisions. It appeared that the filter paper method did not work. Cells came through the filter paper, but there was little selection for small cells.

Before the filter-paper synchronization method was discarded, a subconscious intervention occurred. The development of the baby machine is related to a dream eerily reminiscent of Kekule's vision of snakes rolling about with their tails in their mouths—the famous inspiration for the structure of benzene. The dream occurred at a Biophysical Society meeting where Helmstetter talked about his work. During a hallway conversation someone asked Helmstetter how long the cells were filtered. He answered, ''a few minutes''. Someone in the group commented ''then the cells must be growing in the filter''. This conversation did not go further that day. But that night, as Helmstetter lay in bed in the dimly lit hotel room, he stared at the ceiling. He began to think about things being attached to the ceiling. Soon the image of

chickens attached to the ceiling appeared in his thoughts. The image then changed to chickens laying eggs. As these chickens laid eggs, the eggs would fall down. Helmstetter suddenly realized that rather than percolate cells through the filters all he had to do was bind cells to the filter paper. If bacterial cells were attached to the filter, they would release newborn cells by division, just as eggs left the hanging chickens. The method was tried with filter paper with success. But an even better method was developed as filter paper gave way to nitrocellulose filters.[4] Now the bacteria were attached only to the surface of a nitrocellulose membrane. The bound cells grew, divided, and released newborn cells that produced an exquisitely synchronized bacterial culture.

Synchronization of eukaryotic cells

There is an enormous literature on synchronizing mammalian cells. Although some selection methods such as elutriation selection (based on cell size) and mitotic selection (based on release of adherent cells from the substrate) have been used occasionally, neither of these methods has had the popularity of non-selection methods such as G_1-phase arrest, double-thymidine blocks, temperature-sensitive mutant arrest, and mitotic blocks using nocodazole. Part of the appeal of these inhibition methods is that the growing cells could be simply treated to arrest cells at what was believed to be a point in the cell cycle. Upon release from the arrest condition, the cells would then produce a synchronized culture. It was believed that these methods could produce a large number of synchronized cells thus simplifying the biochemical analysis of the cell cycle. The common theme of these non-selection or arrest/release methods is that the cells are inhibited by either environmental or chemical means and it is assumed that the inhibited cells are arrested at a particular point in the cell cycle.

Rarely, if ever, are cell-division patterns presented to support the proposal that arrested/released cells are synchronized. Besides the enormous investment in labor due to the long interdivision times of eukaryotic cells, one of the main problems with presenting cell division patterns is that synchrony using these methods rapidly dies out. Sometimes it is said that only one cycle of synchrony can be observed before the synchrony decays. The main explanation given for the rapid decay of synchrony is that eukaryotic cell cycles are very variable. Cumulative variation, it is argued, leads to the rapid decay of synchrony.

A completely different explanation of synchrony decay appears when we see the eukaryotic baby machine in action.

The development of a eukaryotic "baby machine"

After more than 15 years of struggle, disappointment, frustration, hard work, and then ultimate success, a eukaryotic "baby machine" has now been developed. The method, again emanating from the laboratory of Charles Helmstetter, is described in a low-key, understated article in *Biotechniques*.[1] The very simplicity of the method may lead to its being overlooked in the rush to analyze the cell cycle using approaches that are more complex. But the method should not be missed. The baby machine method may well revolutionize the study of the mammalian cell cycle.*

As with the bacterial baby machine, the eukaryotic baby machine works by binding exponentially growing cells to a membrane. Throughout the binding process (which takes only a few minutes) the cells are kept in warm medium and are never subjected to any harsh changes in temperature or medium that could lead to unwanted perturbations. After inverting the apparatus, fresh, warm medium is pumped through the membrane. Cells grow normally on the membrane as indicated by the normal doubling time of cells bound to the membrane. Newborn cells are eluted as indicated by their narrow size distribution (determined either by laser light scattering or Coulter Counter sizing), and their DNA distribution. But the essential proof that the cells eluted from the membrane are a collection of newborn cells is the pattern of synchronized cell divisions produced by these cells.

An example of synchronized growth from cells produced by membrane-elution is shown in Fig. 1 (taken from Ref. 1). It is the cell number graph in Fig. 1 that is most revolutionary. I have been looking at eukaryotic cell cycle studies for over 35 years and I have never seen a synchrony graph like this. There are four clear cell cycles. Observe that no lines are drawn through the points—*res ipsa loquitur*—the data speaks for itself. DNA analyses of various fractions (Fig. 1) indicate that the cells exhibit the proper DNA contents at the appropriate times during the division cycle. In particular, observe that the cells have G_1-phase DNA contents at the start of three successive cell cycles. There is only a smattering of G_2-phase DNA content cells in the sample from the start of the third cycle, and these cells may be due merely to sampling the cells before all cells have divided. The final touch is the demonstration that the cell size distribution of the synchronized cells is narrower than the cell size distribution of the original population, and cell size changes as expected for synchronous growth (Fig. 1). Finally, the baby machine method is simple to perform and very reproducible.

*In the interest of full disclosure, I point out that I have been a friend of, and collaborator with, Charles Helmstetter for almost 40 years since we met years ago in Ole Maaløe's laboratory in Copenhagen. I have reviewed this history in an article celebrating the 30th anniversary of the bacterial "baby machine" and the application of the baby machine methodology to the study of DNA replication during the bacterial division cycle.[6] So, if one wishes to temper my enthusiasm with a bit of skepticism as to the revolutionary nature of the baby machine method, be my guest. But such skepticism runs the risk of missing a truly wonderful technological development.

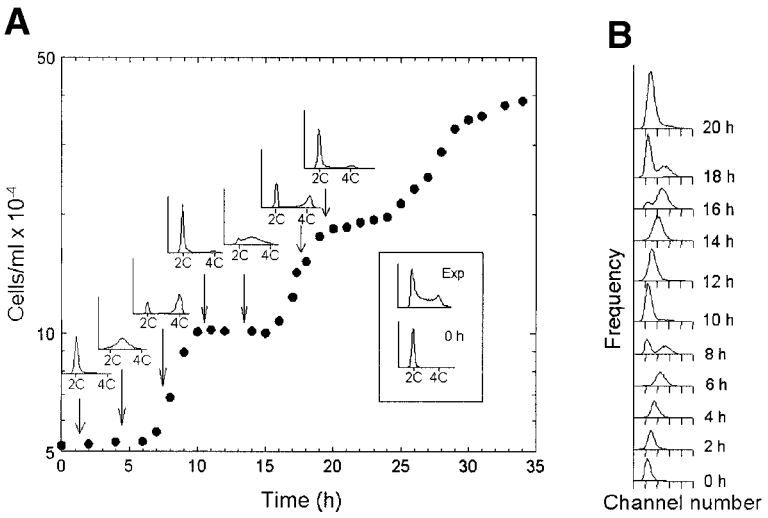

Figure 1. Synchronous growth of newborn L1210 cells (taken from Thornton M, Eward KL, Helmstetter CE. Biotechniques 2002;32:1098–1105 with permission of Eaton Publishing Company). **A:** Points of synchronous growth in a culture of newborn cells collected for 30 minutes from a baby machine. DNA distributions were determined by flow cytometry at the cell cycle ages indicated by the arrows during synchronous growth. The inset shows a comparison of the DNA distribution in an exponential-phase culture to that in a sample of the effluent. **B:** Cell size distributions during synchronous growth of L1210 cell samples taken at the indicated times from the synchronously growing culture shown in (A).

Implications of a eukaryotic baby machine

The baby machine method has substantive implications for understanding the cell cycle simply because three synchronized divisions are observed. As noted above, when cell synchronization has been tried in the past using primarily starvation/arrest/release methods, it was usually found that, after a first cycle, there was a rapid loss of synchrony or no synchrony by the second cycle.[7] Rapid synchrony decay has been attributed to normal mammalian cell-cycle variability. The absence of successive synchronized cell cycles was accepted as the normal pattern to be expected for synchronized cells. This explanation proposes that simple stochastic variation prevents multiple synchronized divisions.

Without the results of Fig. 1 in hand, no refutation of the cell-cycle variability explanation was available. But with the results in Fig. 1 available—a new "gold standard" for synchrony—it is possible to suggest that the reason that no second, third, or fourth cycles of synchronized division are observed with arrest/release methods is that the cells proposed to be synchronized by these treatments are not actually synchronized. In support of the experimental critique of starvation synchronization is a theoretical analysis proposing that inhibited cells are not, and cannot be, synchronized.[8] Thus, the synchronous growth pattern for baby-machine cells is an experimental illustration of the correctness of the theoretical proposal that forced synchronization cannot synchronize cells.

The future of mammalian cell cycle studies

We now have a method that can allow the investigation of the cell cycle of cells that are minimally disturbed and that synchronously pass through the division cycle. For proteins synthesized at a particular time during the division cycle, it should now be possible to line up the expression and synthesis times to make a map of cell-cycle-specific syntheses. If various structures are produced in a certain order during the cell cycle, it should be possible to use electron microscopy on these synchronized cells to get the normal pattern during the division cycle.

Above all, the baby machine method is one that should lead to a study of the cell cycle that is devoid of artifacts that could be introduced by harsh treatments of cells such as starvation and inhibition.

The existence of the baby machine for eukaryotic cells is a wonderful result in itself. It is now expected that more substantive results will come from the direct study of these synchronized cells.

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