Reply: whole-culture synchronization – effective tools for cell cycle studies

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Studies of gene expression during the eukaryotic cell cycle in whole-culture synchronized cultures have been published using many methodologies. These procedures alter the state of the cell cycle for a population of cells, rather than purifying a population of cells that are in the same state. Criticism of these methods (e.g. see Cooper, this issue, pp. 266–269, DOI:10.1016/ j.tibtech.2004.04.009) suggests that these studies are flawed, and posits that such methodologies cannot be used to study the cell cycle because they alter the size and age distributions of the cultures. We believe that whole-culture cell cycle studies work even though they alter the size and age distributions: these cells still progress through the cell cycle and although we do not suggest that the methods are perfect, we will explain how these microarray studies have successfully identified cell cycle regulated genes and why these results are biologically meaningful.

The cell division cycle has long been a focus of molecular research into the nature of eukaryotic cell biology. Studies of gene expression during the cell cycle are among the earliest results obtained from northern blotting, which measured mRNA levels as a function of cell cycle state in the sea urchin [1]. Genome-scale technologies have identified an even greater number of genes whose transcription is periodically regulated throughout the cell cycle [2,3], most recently obtaining whole genome results [4,5] using DNA microarrays.

There are many published methods for achieving synchronized cell populations, including chemical interference, physical separation and genetic restriction. Most of these methods take a population of cells of varying 'ages', as measured by time since last cell division, and attempt to force the cells into one particular cell state, usually by restricting cell-state progression past a certain point (e.g. use of hydroxyurea to block DNA replication). Cells become arrested at that point and are subsequently released from the arrest in an effort to get one or more rounds of synchronized cell division. In almost all cases batch synchronization methods allow cells in a population to continue growing during the arrest and it is not uncommon for some cells to reach volumes several times their normal division volume.

In the previous Opinion article by Stephen Cooper (this issue, pp. 266–269, DOI:10.1016/j.tibtech.2004.04.009)

batch synchronization methods for cell cycle experiments are compared to a perpetual-motion machine, as an idea in violation of a fundamental principle. He argues that methods starting with cells of differing ages are incapable of transcending this barrier because even though they might be blocked at the same point, their differing ages mean that they are in different cell states. We do not dispute that whole-culture synchronization fails to make the age and size distributions uniform, in fact it necessarily skews both from what is observed in steady-state culture. What we do dispute is the idea that removal of these biases is necessary to study the cell cycle, and in doing this we highlight the ample experimental evidence that shows the effectiveness of whole-culture synchronization.

Experimental support for batch synchronization

There are five main pieces of evidence that show that batch synchronization of whole cultures has resulted in justified conclusions about cell cycle gene expression. First, at least in yeast, the cell cycle of synchronized cells appears normal. Second, large numbers of cycling genes have been identified in a range of eukaryotic organisms for which a homolog is periodically expressed in another species. Third, genes whose transcripts have been identified as periodic frequently have functions consistent with the genes' time of expression – indeed, mutants of many of the essential genes for which transcription has been demonstrated to be periodic arrest at the point in the cell cycle at which their transcript normally peaks. Fourth, there is substantial agreement as to which genes have periodically regulated transcripts between different batch synchronization experimental systems, as well as with non-batch synchronization methods. Finally, and perhaps most importantly, because it shows that such genes exist, several genes that cycle in whole-culture synchronization show cell-cycle-dependent gene expression when examined at the level of single cells.

There is ample evidence that cell populations that have been synchronized by a batch synchronization method retain several properties that would be expected of an unperturbed cell undergoing normal cell division. Early microscope studies of yeast clearly delineated landmarks of the yeast cell cycle that occur in unperturbed cells. In yeast, a small daughter cell first grows to a critical cell size, and then at the point called START, commitment to the next division is made, whereby removal of adequate

nutrients is unable to prevent cells from entering the cell cycle [6,7]. Immediately following START, the spindle pole body is duplicated [8,9] and a small bud emerges. After replication, the chromosomes are separated in a spindle that extends into the new bud, and finally the cells divide after completion of telophase. In a mixed, asynchronous population of yeast cells, all of these states are clearly visible. After batch synchronization, cells released from arrest show all of these landmarks in exactly the same order, and with approximately the same timing. Furthermore, it can be observed, often for several cell cycles, that most of the cells are reaching these landmarks at approximately the same time, as adjudged by fluorescence-activated cell sorting (FACS) analysis, budding index, and staining of the spindles. Thus, although we do not suggest that batch synchronization produces identical cells, with identical kinetics of cell division (we liken achievement of that state experimentally to a perpetual-motion machine), we state as fact that cells dividing after batch synchronization undergo cell cycle events that occur in unperturbed cells.

The cell cycle of two eukaryotes, *Saccharomyces cerevisiae* and *Homo sapiens* (using human cell lines), have been analyzed using whole-genome transcriptional profiles generated from batch-synchronized cultures. In each case, several experiments were carried out, with at least three different methods of batch synchronization used for each organism. In both the studies of Spellman *et al.* of *S. cerevisiae* [5], and of Whitfield *et al.* of *Homo sapiens* [4], at least two measures of cell synchrony were used to judge how synchronous the cell populations were during their experiments. Though it is difficult to summarize these data, we have taken the liberty of showing gene expression profiles for genes from our own work using ratiograms (Figure 1). This shows that batch synchronization conditions can yield gene expression data that cycles, using different methods of synchronization. Many of the cycling genes have homologs in another species that also cycle providing evidence that the batch synchronization methods are working. Figure 1 also shows cell cycle expression of four sets of homologous genes (PCNA, Histone H4, MCM4, Cyclin B), which are expressed during distinct (and largely equivalent) portions of the cell cycle. An additional line of evidence from human studies was that many of the periodically expressed genes were present at a higher level in actively proliferating cells, compared with cells having a lower mitotic index [4]. This phenomenon is in concordance with expectations for genes that are periodically expressed in the cell cycle.

Some would argue that the previous results are insufficient; it is plausible that biological properties not dependent on the cell cycle could cause expression in a cell cycle-dependent manner, based solely on the method of synchronization. A compelling counter-argument is that a large fraction of cell cycle transcribed genes have biological roles in the cell cycle. For example, all of the genes shown in Figure 1 are crucial for proper cell cycle functioning. More importantly, those genes that are cell cycle regulated and are involved in the cell cycle are nearly universally transcribed just before the point in the cell cycle at which that gene's product is needed. Thus, as has been noted

frequently in the past, gene expression and gene function are often tightly coupled. A second compelling counter argument is that most of the genes show similar or identical periodic expression following release from different arrest points, using different synchronization methods.

Cooper's opinion stresses the advantages of the 'baby machine' method, in which a population of cells is collected for which the age since cell division is arbitrarily small. Although a 'baby machine' for yeast has been described [10], no eukaryotic cell cycle has yet been studied using a 'baby machine'. However, many genes that have been shown to have periodic transcription in yeast were done so using elutriation, which is a selective, rather than restrictive batch synchronization method. Elutriation isolates the smallest cells from a culture, which for yeast are typically G1 daughter cells, and thus of the same, or comparable age to each other (indeed, elutriation has been referred to as a 'baby machine' [11]). Further, many of these elutriation-identified genes are also identified by other batch synchronization methods, showing that restrictive batch synchronizations generally agree with selection methods such as elutriation (e.g. see [12–16]). One group has produced cell cycle gene expression data based on a design that is essentially that of a 'baby machine', using the bacteria *Caulobacter crecentus* as a model [17]. It is difficult to compare and contrast the data from *Caulobacter* with the data from yeast and human, but it is instructive to note that many broad classes of genes, particularly between yeast and *Caulobacter*, are cell-cycle-dependent in each set (e.g. DNA replication, DNA repair, cell division, chromosome segregation, cell wall and/or membrane genes). Importantly, *Caulobacter* genes display the same property of peak gene expression just before the point in the cell cycle when their product will be needed.

Perhaps the most important response to critics of batch synchronization is the concordance between protein expression localized in individual live cells and mRNA measurements. These are particularly difficult experiments, particularly in yeast given their small size but it has successfully been done for a small number of proteins and mRNAs. A few examples of this (from *Saccharomyces*) are SLK19, HSL7, CLN2, and DBF2, which show related patterns of mRNA expression and protein expression [1,18–20]. Even more strikingly, the mRNA levels of ASH1 have been imaged *in situ* in individual cells and correlate with the observed pattern of gene expression by batch synchronization [4,20]. Finally, the initial rounds of embryonic cell division (typically *Xenopus* or sea urchin embryos) are an excellent model of the cell cycle that involve populations of inherently synchronously dividing cells that have not been synchronized by an external method, and in which cycling genes homologous to those shown in Figure 1 have been identified.

How do we perform high quality batch synchronization?
Now that we know it is possible to derive reasonable data from batch synchronizations, we might ask how do we know if batch synchronization was successful and that the resulting data are meaningful? Perhaps the first rule
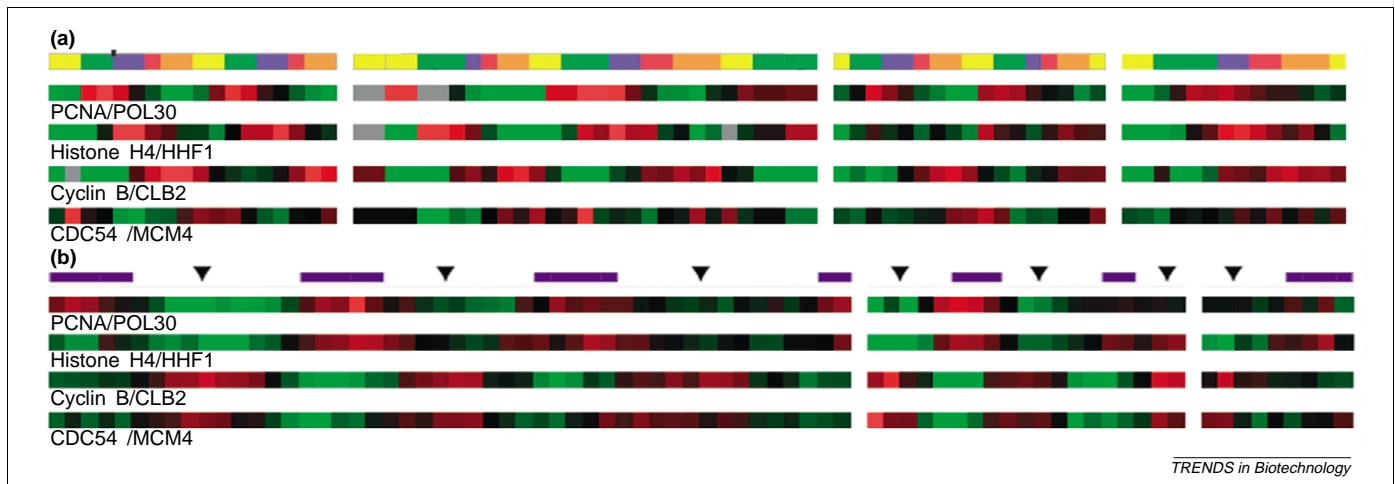
Figure 1. Gene expression ratiograms of cycling homologous genes. Expression profiles for four pairs of homologs from **(a)** *Saccharomyces cerevisiae* and **(b)** *Homo sapiens* are shown. Four experimental series are shown for yeast and three are shown for human. Data are taken from web supplements (genome-www.stanford.edu). Changes in gene expression are proportional to intensity with increased expression from the mean in red and decreased expression from the mean in green. Black represents the mean expression for each gene in each time series. Phases of the cell cycle in (a) are indicated by colored bars over the experimental time series M/G1 (yellow), G1 (green), S (purple), G2 (red), and G2/M (orange). In (b) S phases are marked by purple boxes and mitosis is marked with arrowheads.

should be, 'do not to trust a single method', although even this is probably too simple.

Overall, well-designed batch synchronization must address disruptions to normal biological processes caused by the manipulations. For examples of manipulations that can cause artifacts, common yeast methods employ shifts to high growth temperatures in a temperature-sensitive cell cycle mutant, which will induce stress responses as a byproduct, or exposure to α factor, which blocks the cells just before the start in the cell cycle. Human cell line synchronizations can use the tubulin depolymerizing drug nocodazole, which will disrupt any biological process dependent on microtubules. We have observed, as expected, that stressed yeast cells produce heat shock proteins, α factor-exposed cells initiate the pheromone response pathway and we might expect tubule-disrupted cells to increase tubulin production. Accordingly, we would not expect tubule-disrupted cells to produce mating genes, or α factor-blocked cells to synthesize tubulin, which, not surprisingly, does not occur in cells synchronized by those methods. Obviously biologists must be aware of the manipulations they perform, and when genes cycle or fail to cycle we can understand that the biological networks can be intertwined and that the behavior of these genes is perfectly reasonable.

We view the cell cycle microarray data as a body of work, which together are self-reinforcing. On its own, no single experiment is adequate to comprehensively characterize the cell cycle transcription of all genes in an organism, but taken together, we believe that they demonstrate unequivocally the periodic transcription of a large number of genes. In no way do we suggest that the data that have thus far been generated are perfect. There are certainly imperfections, in both the technical aspects of the experiments, as well as the subsequent analyses of the data, and undoubtedly it is possible to generate better data through careful experimentation, and to better analyze it. A 'baby machine' might indeed allow this, although we are still waiting for such data to be generated for comparison purposes. Of course science thrives on open discussion, and we applaud Cooper for standing up and saying that the Emperor has no clothes. In this case, however, we don't agree with him.

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