

Rejoinder: whole-culture synchronization cannot, and does not, synchronize cells

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There have been numerous proposals suggesting that whole-culture methods – in which all cells in a growing culture are treated identically – can synchronize cells. An explicit defense of these methods has been presented (Spellman and Sherlock, this issue, pp. 270–273, DOI:10.1016/j.tibtech.2004.04.010). Here, this defense of whole-culture ‘synchronization’ is subjected to a critical evaluation leading to the conclusion that whole-culture synchronization cannot synchronize cells – at all. Whole-culture methods cannot produce a set of cells that reflects the size and genome composition of cells of any particular cell-cycle age during the normal cell cycle. Thus, in addition to the well-recognized problem of artifacts, it is proposed that experiments using whole-culture treatments (usually starvation or inhibition methods) are not suitable for cell-cycle analysis because these methods do not produce a synchronized culture.

The response of Spellman and Sherlock to my proposal that whole-culture or batch synchronization cannot synchronize cells is clearly and strongly written (both articles in this issue; pp. 266–269, DOI:10.1016/j.tibtech.2004.04.009, and pp. 270–273, DOI:10.1016/j.tibtech.2004.04.010). It represents the ideas generally held to support the contrary idea, that whole-culture approaches can produce a synchronized culture.

The main problem with their response is that the article does not consider or refute the detailed analysis put forward as to why such whole-culture methods do not work. The key paper presenting this analysis is entitled ‘Rethinking synchronization of mammalian cells for cell-cycle analysis’ [1]. This paper is a rigorous presentation of why such methods do not work, although the idea was presented in earlier papers in a more intuitive manner [2–4].

The analysis presented in [1] showed that the widely used whole-culture synchronization methods (e.g. serum starvation, mass arrest, DNA arrest, mitotic arrest etc.) do not narrow the size distribution of cells. More generally, it is shown in Figures 1 and 2 of a review on retinoblastoma protein phosphorylation [4] that whole-culture synchronization does not produce cells with a uniform state or condition. In this paper [4] also note the section ‘An industrial analogy to the cell cycle’ for another explanation of why whole-culture methods do not work. Whole-culture

‘synchronization’ methods do not produce a population of cells that reflect the size and DNA content or mitotic configuration, or the physiological state, of cells of a particular cell age during the cell cycle of cells growing in steady-state, unperturbed, conditions. Spellman and Sherlock agree with this point, as they state: ‘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.’

The purpose of synchronization is to produce cells that enable one to study the ‘normal cell cycle’, the cell cycle of cells growing in medium in unperturbed conditions. Synchronization is merely a necessary step to obtain enough cells of a particular cell age to perform chemical measurements. Whole-culture synchronization might yield results and data, but none of this data are related to the pattern of events or gene expression patterns during the normal cell cycle because whole-culture synchronization does not produce a collection of cells that mimic the size and DNA configuration of cells of any specific age during the normal cell cycle.

There are two important distinctions that must be made. First, it is important to distinguish the argument about whether whole-culture synchronization can synchronize cells from a different question, whether or not there are genes expressed in a cell-cycle-specific manner. It is possible to accept that there are some or even many genes expressed in a cell-cycle-specific manner and yet demonstrate and accept that whole-culture synchronization does not work. Spellman and Sherlock refer to numerous experiments asserting the existence of cell-cycle-specific gene expression (this issue, pp. 270–273, DOI:10.1016/j.tibtech.2004.04.010). It is not valid to conclude that the existence of genes expressed in a cell-cycle-specific manner supports the validity of whole-culture synchronization methodology.

Second, it is important not to equate the existence of ‘cell cycle functions’ with proving or accepting that these genes are expressed in a cell cycle-specific manner or that the existence of these genes proves that whole-culture synchronization does synchronize cells. For example, by definition we can say that DNA polymerase has a cell cycle function. This enzyme presumably functions during the DNA-synthesis phase of the eukaryotic cell cycle, the S phase. But merely because this cycle-specific function exists and is associated with a particular gene product

does not automatically mean that this gene is expressed in a cell-cycle-specific manner. One could have DNA polymerase made continuously throughout the cell cycle, yet have it function only at a particular time during the cell cycle in response to some signal that starts S phase.

Spellman and Sherlock defend whole-culture synchronization by accepting that whole-culture methods are not perfect. They argue that these imperfections should not get in the way of using these methods to study the cell cycle.

Let me make it perfectly clear – I am not arguing that whole-culture synchronization is merely a poor method of synchronization, or that such whole-culture or batch methods do not produce good synchronization. Rather, I argue that cells produced by such whole-culture methods are not synchronized at all [1]. Cells produced by whole-culture approaches are not poorly synchronized, or weakly synchronized, or imperfectly synchronized; these cells are not synchronized at all.

I agree with Spellman and Sherlock that after such whole-culture treatments one can get, and in fact one does get, varying patterns of gene expression. However, one should not use varying or even cyclical patterns of gene expression to support synchrony. The defect in this argument is that it is assumed that the cells are synchronized, and therefore any patterns of change observed after release from an arrest condition are related to the cell cycle. It is equally possible, and even most likely, that the cyclicities observed are the result of unwanted and undesirable perturbations introduced by whole-culture treatments. The existence of numerous false positives [5] in the yeast data [6] is an indication that whole-culture methods can induce perturbations in gene expression.

An interesting point relates to the use of 'known' cell cycle genes as evidence that whole-culture methods work. With regard to the yeast experiments [6], an examination of almost all of the available papers on prior proposals of cyclic gene expression [5] indicates that the vast majority of the previous analyses were based on α -factor synchronization, temperature-sensitive-arrest synchronization, raffinose–galactose arrest-regrowth, feed-starve synchronization, nocodazole synchronization, and hydroxyurea arrest – all whole-culture methods – with only a small fraction of the papers using selective methods such as elutriation. Thus the repetition of cyclicity in the microarray analysis is probably a result of the use of the same methods that were used to 'synchronize' or align cells in the previously published papers. The microarray results [6] merely present a confirmation of the variations (perturbations?) previously found using classical assay methods.

A problematic analysis is exemplified in the reference to published results on microarray analyses of gene expression during the cell cycle. Spellman and Sherlock write: 'Although we do not suggest the methods are perfect, we will show how these microarray studies have successfully identified cell cycle-regulated genes.' Two studies in particular [6,7] are cited as supporting the conclusion that one can use whole-culture synchronization to identify cell-cycle-specific gene expression.

The results of Whitfield, *et al.* [7] are a striking example of an incorrect conclusion that whole-culture methods can

synchronize cells. Two whole-culture methods were used, a double-thymidine block and a thymidine-nocodazole block. Flow-cytometric analyses of DNA patterns following release from these blocks are presented to support the conclusion that the cells are synchronized. The DNA patterns from Figure 1 of Whitfield, *et al.* [7] clearly show that the cells are not synchronized. The initial cells have a DNA content that is never repeated in subsequent time points. Further, there is no clear passage of the cells through successive phases of the cell cycle. Merely saying that the cells produced by these whole-culture methods are synchronized does not make it true.

Before one despairs of seeing good synchronization results, I can refer the reader to results that do fit the expectations for synchronization [8–10], although these results are due to the membrane-elution or the 'baby-machine' method, a selective and not a whole-culture method. (For a description of the membrane-elution method and the results obtained, the reader can go to hyper.fit.edu/biology/Files/Helmstetter.pdf). One should compare the flow-cytometric analyses of cells synchronized by membrane-elution with those of Whitfield *et al.* [7] to see why one should not accept these whole-culture methods as producing synchronized cells. The flow cytometric analyses of cells produced by membrane-elution fit the criteria for synchronized cells, whereas the flow cytometric analyses of the cells produced by whole-culture synchronization (double-thymidine block or thymidine-nocodazole block) do not fit the criteria for synchronized cells.

The yeast microarray results of Spellman *et al.* [6] have been analyzed [5] with results indicating that there are perturbations caused by the three whole-culture methods used to 'synchronize' cells. The cyclicity values for all of the four experiments (three whole-culture and one elutriation) were not reproducible. Further, the phase locations that did show some measure of reproducibility within the whole-culture methods did not reproduce with the selective elutriation method, arguably the least perturbing method. This result implies that the different whole-culture methods merely produce similar perturbations. A detailed analysis of individual genes showed that over the four experiments there is an enormous amount of non-reproducibility of cyclicity [5]. In passing, it should be noted that the characterization of elutriation as a 'baby machine' method should be avoided. Elutriation is a 'selective' method that isolates cells of a certain sedimentation coefficient. The sedimentation coefficient is related to both size and shape of the cell. By contrast, the membrane-elution method isolates cells on the basis of cell age, and produces a selection of newborn or 'baby' cells [8–10]. I recommend that elutriation be called a 'hydrodynamic' method.

An interesting argument used to support the use of whole-culture synchronization methods is given as follows: 'An additional line of evidence from human studies was that many of the periodically expressed genes were shown to be present at a higher level in actively proliferating cells, compared with cells with a lower mitotic index... This phenomenon is in concordance with expectations for genes that are periodically expressed in the cell cycle.' I suggest

that this conclusion is a *non sequitur*. Just because genes are highly expressed in growing cells and found at lower levels in non-growing cells does not have anything to do with whether genes are expressed in a cell-cycle-specific manner.

Another argument raised is: 'Some might 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.' As I have noted at the start of this analysis, one must distinguish between genes that have a 'role in the cell cycle' and the pattern of gene expression. In the example given above, one could imagine DNA polymerase being made continuously during the cell cycle, yet define this as a protein with a 'role in the cell cycle.' Genes involved in cell cycle events might not be expressed at particular times during the cell cycle. Finding such cycle-specific expression using methods that do not synchronize cells does not indicate that such cell-cycle-specific expression occurs in normal, unperturbed, cells.

Regarding the general body of microarray studies of gene expression during the cell cycle, Spellman and Sherlock propose: 'On its own, no single [microarray] 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'. An answer to this argument is presented in a review of the extant set of microarray analyses of gene expression during the cell cycle of different cell types [11]. The general conclusion of this review is that there are no microarray experiments that qualify as an unequivocal demonstration of particular genes being expressed in a cell-cycle-specific manner (<http://www.cellandchromosome.com/content/2/1/1>). But even more to the point, as noted at the start of this article, the mere demonstration of 'the periodic transcription of a large number of genes' is no support for the idea that whole-culture methods produce synchronized cells.

Above all, one should refer to the criteria for synchronization as given in Box 1 of the article in this issue on pp. 266–269 (DOI:10.1016/j.tibtech.2004.04.009) that initiated this debate. The *sine qua non* of synchronization is that cells should pass through the cell cycle as a synchronized cohort and divide synchronously. Without this evidence one can never say that cells in a presumed 'synchronized' culture are truly synchronized. I await a demonstration that whole-culture 'synchronized cells' can fit the criteria proposed in Box 1 of my original Opinion article (this issue, pp. 266–269, DOI:10.1016/j.tibtech.2004.04.009) (e.g. proper size distributions, proper

DNA contents, synchronized divisions etc.) as well as the cells produced by membrane elution.

In conclusion, it is important that researchers who wish to use whole-culture synchronization – certainly the current and dominant approach to cell cycle analysis of eukaryotic cells – take the time to look at the arguments showing that whole-culture synchronization methods cannot synchronize cells [1,3,4,12], that whole-culture methods do not synchronize cells [2,8,13–15], and that whole-culture methods should not be used to analyze gene expression during the cell cycle.

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