

Bilbao, 01-02-2017

Dear Editor,

We are submitting the revised version of our manuscript JoVE55745 entitled “**Studying cell cycle-regulated gene expression by two complementary cell synchronization protocols**”. We have carefully read the comments of the reviewers, and have made the required changes. Here is our point-by-point response to the Editorial and Reviewer’s comments.

We hope that you find our revised manuscript acceptable for publication in *JoVE.*

Yours sincerely,

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**Response to Editorial comments:**

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

* The manuscript has been proofread.

*2. Please define all abbreviations before use.*

All abbreviations have been defined before use

*3. Please use SI abbreviations: h for hours instead of hr (Figure 1, 4B, etc.)*

* We have used SI abbreviations.

*4. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.*

* We have removed symbols.

*5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*

* We have removed symbols. Products are referenced in the Table of Materials.

*6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*

* We have avoided personal pronouns.

*7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.*

* We have moved it to Discussion.

*8. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*

* We have highlighted around 2.75 pages of the protocol.

*9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*

* Highlighted steps form a cohesive narrative.

*10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*

*a) Critical steps within the protocol*

*b) Any modifications and troubleshooting of the technique*

*c) Any limitations of the technique*

*d) The significance with respect to existing methods*

*e) Any future applications of the technique*

*11. The current discussion is more of an original research article discussion when it should focus on the methodology.*

* We have rewritten the discussion section to focus on methodology, and we have included discussion on critical steps of protocol, troubleshooting, limitations and applications.

**Response to Reviewers:**

**Reviewer #1:**

*Manuscript Summary:*

*The authors of this manuscript describe two protocols to synchronize cultures of mammalian cell lines across different phases of the cell cycle. They propose the use of this method for cell cycle -related gene expression studies, such as the analysis of phase-specific transcriptional programs or the study of drug-response mechanisms at the gene expression level.*

*These protocols are widely used in the cell cycle field and, therefore, their detailed description might be of utility for a significant fraction of the scientific community. In general, the protocols are well explained and with sufficient detail, and all materials and reagents required are detailed in the table of Materials.*

*Major Concerns:*

*-The authors claim that Thy-noc protocol is mostly appropriate to study G1 and S phase, whereas HU protocol works better for S, G2 and M phases. However, according to cell cycle profiles shown in Figure 2, a mostly S- phase population is only obtained at 3h release from HU, but not after nocodazole release, as it would also be expected. Could this be due to mitotic slippage in the conditions used for mitotic shake -off? (See also comment on 1.7.1 below). This point should be clarified.*

We agree with the reviewer in that an enriched S-phase population is only observed with HU protocol. By contrast, Thy-Noc protocol is best for enrichment of populations in G1 and S-phase entry, but not so good for S and G2 phases (Fox, MH, 2004, *Methods Mol Biol*, 241:11-16). In the revised version of the manuscript, we have modified the text to clarify this point (page 2, line 46; page 14, line 451 and page 17, line 553).

We believe that the main reason for the lack of an enriched S-phase population is a progressive loss of synchronicity. Although we do not discard that mitotic slippage may happen to some extent, the vast majority of the cell population shows a 2C DNA content upon removal of nocodazole, indicating completion of mitosis and correct progression through the next cycle (please see Figure 2A and Figure 3A of the revised manuscript).

*-Related to the above point it is very difficult to valuate the actual enrichment in each cell cycle phase without showing the percentage of cells in each phase (Figure 2 and Figure 4). Ideally, an EdU and/or BrdU staining should be included to facilitate the identification of S phase cells. On the other hand, a specific mitotic marker, such as pH3 or MPM-2, should also be included in the FACs analysis to identify the percentage of mitotic cells within the 4n population. This is an important control for the time 0 of thy-noc protocol, at which a pure mitotic population should be obtained after a mitotic shake-off. These stainings across the different time points will also allow to study the reversibility of these protocols, and to ensure no cells left arrested in mitosis or S-phase after release from nocodazole and HU/Thy, respectively.*

As pointed out by the reviewer, incorporation of EdU or similar stainings allow the precise determination of cells in S phase. Nevertheless, it increases both, complexity and economic cost especially when large series of time points (e.g. sample collection every 3 h) and conditions (e.g. treatments) are compared. We believe that propidium iodide staining coupled to a FACS analysis software program that discriminates cell cycle phases constitutes an optimal monitoring procedure due to the simplicity of the procedure and to its accuracy in detecting cell cycle phases.

Regarding the population of cells with 4C DNA content, following the suggestion of the reviewer, we have included immunoblot analysis of pH3 levels in cells upon Thy-Noc and HU-based synchronization protocols (Figure 3B of the revised manuscript) and we have performed a new experiment in order to verify the purity of our mitotic population obtained by the Thy-Noc protocol (please note new Figure 2A). In addition, the protocol for pH3 analysis has been included in the Methods section and in the modified Discussion section.

*-Different agents allow cells to be arrested at the G1/S transition such as HU, Thymidine or aphidicoline, among others. Why the authors show thy for the first protocol vs HU for the second? It is known that some of these agents such as HU might induce replicative stress. What's the level of damage induced in the conditions described in these protocols? Is it fully reversible? Does it determine the use of any specific G1/S blocker vs. others?*

As mentioned in the manuscript and pointed out by the reviewer, drug-mediated synchronization protocols are based on transient inhibition of cell cycle progression and therefore, tightly linked to checkpoint activation. Consequently, HU, as well as other inhibitors of DNA replication such as thymidine or aphidicolin, cause replication stress. In fact, this aspect has been previously mentioned by other authors (e.g. Darzynkiewicz *et al.,* 2011, *Methods Mol Biol*.). In order to minimize this limitation, it is crucial to select experimental conditions that are able to stop progression of the cells at some point of the cell cycle, but also to allow adequate progression once the drug is removed. In the new version of the manuscript we have included some of the optimization experiments that we performed with U2OS cells. As shown in the new Figure 2B, 24h treatment with Thymidine was not as good as HU in arresting cells in G1/S transition. Furthermore, although comparable results were obtained by HU and Double Thymidine treatment, a persistent peak in 2C DNA content (G1 phase) was detected in cells released from Double Thymidine protocol while a proper progression towards S and G2 phases was observed upon removal of HU. These data, suggesting a partial but unresolvable DNA damage and checkpoint activation after the removal of the second thymidine round, led us to the selection of the HU-based method for U2OS cells. In addition, progressively decreasing p21Cip1 expression levels (Figure 5B, right graph, of the new version of the manuscript) support the transient nature of the arrest induced by HU as well as the lack of further G2 checkpoint activation. Required changes have been done in the manuscript including a new figure (Figure 2), figure-related representative results (page 11), figure legend (page14) and discussion in the corresponding section (page 16 and 17).

*-The protocols described are exclusively based on U2OS cell line. Validation of the synchronization protocol in at least another cell line should be shown. Moreover, limitations and/or variations of these protocols when apply to other cell lines should also be discussed.*

Synchronization protocols described in this manuscript have been previously employed in several studies applied to cell lines such as HeLa cells (Westendorp et al., 2011, Liu et al., 2011, Oncogene), thereby validating the protocol. Moreover, their limitations have been discussed in the new version of the manuscript.

*Minor Concerns:*

*-Cell cycle synchronization across different phases may also be monitored by Western-blot analysis as shown in Figure 2B. However, the markers shown in the figure are quite limited. Other cell cycle regulated genes such as Cyclin A, pH3 or geminin for instance should be included to better discriminate among cell cycle phases.*

Following the suggestion made by the reviewer, we have added immunoblot data on pH3 levels to the markers previously shown in Figure 2B (now renamed Figure 3B). Cyclin E1 and Cyclin B1 were selected due to their narrow expression window and distinct expression peaks during the cell cycle, in contrast to Cyclin A or D, which show broader expression patterns in U2OS cells and are not optimal for defining cell cycle phases (Asghar *et al.*, 2015; *Nature Reviews Drug Discovery*).

*-FACS software and/or algorithm used for gating cell cycle profiles should be specified (Figures 2 and Figure 4).*

Summit V4.3.01 (Dako) was used for FACS data analysis. This information has been included in the revised manuscript (page 14, lines 445, 450 and page 15, line 477).

*Additional Comments to Authors:*

*Specific notes on protocol steps:*

*1.4. Slight warming might help dissolve thymidine. This could be added as a suggestion/recommendation.  
1.7.1. It is important to be careful in the washing steps following collection of detached mitotic cells in nocodazole-containing medium. That means either use PBS-cold for the washing steps and/or add nocodazole to the PBS used for washing, in order to avoid slippage from the mitotic arrest. This can be monitored by FACs, based on PI+pH3 or MPM-2 staining (as discussed above), since a mitotic slippage would result in a fraction of 4n cells, which are negative for the indicated mitotic markers.*

We thank the reviewer for the comments made in order to improve the protocols described in the manuscript. All recommendations have been included in the revised version (page 5, line 133 and page 17, line 532).

**Reviewer #2:**

*Manuscript Summary:*

*In the present manuscript Apraiz and co-workers combine two broadly utilized cell synchronization protocols (hydroxiurea and a double thymidine+nocodazole) for the accurate RT-PCR mediated analysis of gene expression during the cell cycle. This can be applied both for the study of unperturbed cell cycle as well as to evaluate the impact of potential therapeutic agents. For the latter, and given the limited duration of cell synchrony after release, the authors nicely illustrate the need for the combination of both protocols in parallel to identify gene-expression variations that are a direct consequence of the treatment under study. This concept is often overlooked thereby potentially leading to data misinterpretation as also clearly illustrated by the experimental data in Figure 4.*

*In addition, although both synchronization methods have been extensively used by many laboratories, the authors provide a detailed and clear protocol that will be useful for the optimization of such approach by other investigators. The overall technical quality of the data presented in the manuscript is remarkable but yet I feel that a few modifications could improve the current version.*

*Major Concerns:*

*1. As discussed by the authors, neither of the two methods ensures cell synchrony over an entire cell cycle following release. This is a limitation and justifies the combination of both methods when studying oscillations in gene expression in order to select the most appropriate protocol for the gene under study. To further support their claim it would have been nice to actually show that extending the observation time beyond 15 hrs (Figure 3) failed to provide accurate experimental results due to increased population asynchrony. For instance (Figure 3 top right), would the G1 peak in E2F1 expression be detected if the experimental time was extended in the HU release? Likewise, (Figure 3 bottom left), would the E2F7 decrease in late S be properly detected if the experimental time was extended in the Thy-Noc release? I am not asking for these experiments to be performed, but the authors may have actually performed these experiments as part of the original study. If results are already available it would be nice to include them to further support the author´s claims.*

In agreement with the point mentioned by the reviewer, the original experiment was extended beyond 15 h after release from nocodazole or HU, up to 24 h. However, the 18 h and 24 h time points were excluded from the manuscript due to the clear asynchrony developed by cell populations already at 15 h after release, which was enhanced at later time points. Regarding E2F1 and E2F7 expression profiles at those later time points, we could, in fact, detect a second wave of E2F1 induction and E2F7 reduction. Nonetheless, the amplitude of those expression changes is clearly diminished, which can be at least partially explained by the loss of synchronicity by this time points. In order to complete the information provided in the manuscript, we have included this information in the body of the text (page 12, lines 370 to 375).

*2. Standard deviation is missing in experiments 3 and 4B (top panels).*

Standard deviation has been included in the experiments mentioned by the reviewer.

*Minor Concerns:*

*Whereas the Cyclin E isoform (E1) analysed in the study is clearly indicated in the figures & legends in the "Representative Results" section is only referred to as Cyclin E. This should be corrected.*

Information regarding CyclinE1 has been corrected in the revised manuscript.