Dear Dr. Sun,

Thank you very much for reviewing our manuscript entitled: "Effects of Deuterium Oxide on Cell Cycle and Vesicle Speed in RBL-2H3 Cells" by Roshni Kalkur et al. (Manuscript #2014:06:2271:0:1:REVIEW). We appreciate the referees' comments and their concerns. We believe that by responding to the various questions and concerns expressed by the referees the revised version is considerably improved. Changes within the manuscript (file name: *tracked_changes.pdf*) and responses to the reviewer's comments presented below are written in red. We also uploaded a clean untracked TeX version as primary file.

Firstly, we would like to address a main concern of reviewer 1 and you that additional experiment are needed to confirm whether the cells enter mitosis or apoptosis.

We thank Reviewer 1 for pointing out the possibility of cells entering apoptosis. Since the funding for this project has ended we cannot perform additional experiments to investigate this in detail at the moment. Therefore, we went carefully through the text and removed all claims that cells halted near the G2-M-phase. Changes were made in the abstract and discussion as well as wording was changed from "cell cycle" to "cell growth". We removed the section called "Effect of D2O on the RBL-2H3 cell cycle". The discussion of the flow cytometry data including the possibility of cells entering apoptosis is now added to "The effect of deuterium oxide on RBL-2H3 cell growth and viability" section, starting on top of page 5.

Secondly, we would like to address the other main concern about the data interpretation for microtubule dependent vesicle transport.

Reviewer 1: "The authors conclude that the colchicine effect in D2O treated cells shows that transport/secretion is microtubule dependent. However, they do not comment on the failure of colchicine to alter the same parameters in cells grown in H2O. So, the readers asks, does this mean that vesicle transport in normal media is not microtubule dependent? The microtubule-dependence of vesicle transport is documented in the literature they cite (Smith et al., 2003). So, one wonders if there is a flaw in the experiment design/results such that they do not detect an effect of colchicine under normal circumstances."

As pointed out in the manuscript, we measured vesicle hop speed for all dil-labeled membrane bound objects that are within the resolution limit of the TIRF microscope. These vesicles can be secretory (undergo microtubule-dependent transport) or not. This is an obvious experimental flaw we thought we clearly pointed out in the manuscript. We added wording to make this clearer at the end of section "Effect of D2O on vesicle motion in RBL-2H3 cells", starting at the end of page 7 before conclusions.

We have taken the reviewer's advice and now added a discussion on why we did not measure a decrease in average vesicle hop speed in colchicine treated cells grown in H2O, see second paragraph on page 7. Since we report on an ensemble average that includes both microtubule-dependent and microtubule-independent transport, the colchicine treatment may have different effects on these two subpopulations. Microtubules extend from the nucleus to cell surface. The disruption of microtubules results in a loss of this structures that may alter the transport of non-secretory vesicles. Once this structure is disrupted, these vesicles may be able to move more freely, which results in an increase of hop speed for this specific population. This change in vesicle speed for non-secretory vesicles can counteract or dominate over the expected

decrease in microtubule-dependent vesicle speeds. By culturing cells in D2O, it appears that microtubule-stabilization is the dominant factor for increasing vesicle hop speed, since colchicine treatment results in a decrease of the ensemble vesicle speed.

Reviewer 2: “For the Figure 3, I cannot tell what’s for mean value for each treatment? Box with red line is the mean value? If yes, any difference between four groups?

Figure 3 is a box plot. A box plot does not typically indicate the mean. The mean values are discussed in the text before figure 3 is mentioned. We did state in the text (and figure caption of Fig.3) that red lines represent the medians for each distribution. But we failed to compare these medians between the four groups. We thank the reviewer for this comment and now added a comparison of the medians between each group, see bottom of page 7 and caption of Fig.3.

Further responses to reviewer’s comments:

Reviewer 1: “The entire DNA staining histogram for day 4 in D2O, is left-shifted from the day one cells. This is ignored.”

We thank Reviewer 1 for noting this. This shift is due to daily fluctuations in the detection system. Due to the reviewer's comment, we noticed that we mixed data from different experiments in Fig.2. This is why the shift occurred in the D2O data put not in the H2O data. We now fixed this mistake including a x-axis scaling mistake. Moreover, to present the data more clearly, we now applied a moving average to smooth the data and normalized all histograms to unit area. Now both D2O and H2O samples experienced a slight shift to the left during a 4 day period. The shift is about the same magnitude for both experimental conditions and therefore we attribute the shift to fluctuations in the detection system. We included corrected figure 2 and this discussion in the text, see end of second paragraph on page 5.

Reviewer 1: They authors make assertions by way of explanation that have no associated rationale or justification. A. On P10 "A possible mechanism for the observed cell cycle arrest in RBL-2H3 cells cultured in D2O is microtubule stabilization. In this case, vesicles that are transported in a microtubule-dependent manner should experience different transport speeds in untreated and D2O treated cultures. As shown by Smith et al. (2003) during mast cell activation via IgE-FceRI crosslinking, secretory granules are transported in a direct and microtubule-dependent manner towards the cell plasma membrane for exocytosis". The question here is, why should microtubule stabilization lead to differences in transport speeds? Please explain this to the reader.

We thank Reviewer 1 for pointing this out and we now added a paragraph to explain this rational to the reader. See first part of section “DiI-stained RBL-2H3 cells for vesicle tracking” at the bottom of page 5 and top of page 6.

Reviewer 1: “Minor issues” have been editorially addressed.

Other issues: We now added info about the localization uncertainty for individual trajectory positions, which is within 100 nm, see page 4, section “Single-vesicle tracking”.

Finally, we thank Reviewer 2's positive remark that our manuscript presents very interesting data and hope that with the submitted content revision our manuscript can now be accepted for publication in PeerJ.

Thank you for your consideration.

Sincerely, Kathrin Spendier
Assistant Professor
Department of Physics and Energy Science
UCCS Center for the Biofrontiers Institute
University of Colorado, Colorado Springs