Dear Dr. Sun,

Thank you very much for reviewing our resubmitted manuscript entitled: "Effects of Deuterium Oxide on Cell Growth and Vesicle Speed in RBL-2H3 Cells" by Roshni Kalkur et al. (Manuscript #2014:06:2271:1:0:REVIEW). We appreciate the referees' comments on the revised manuscript. Based on these comments we made further improvements to our manuscript. 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.

A) Firstly, we appreciate the persistence of Referee 1 regarding missing clarification on the colchicine effect on non-D2O treated cells. Answers A1, A2, and A3 below address this concern.

A1) As pointed out in the text, we classified vesicles with low speed (less than 10^{-2} µm/s) as stationary structures. These vesicles were not included in our analysis. Since colchicine is expected to disrupt microtubules, the proportion of stationary vesicles should increase. We went back and looked through our data to determine the proportion of such stationary vesicles in H2O and D2O cultures with and without colchicine treatment. In H2O cultures the proportion of stationary vesicles increased from 0.043 for normal cultures to 0.418 in colchicine treated cultures. A similar trend was observed for D2O cultures for which the proportion of stationary vesicles increased from 0.065 to 0.580. This analysis shows a clear colchicine effect on non-D2O treated cells. We now added this result into the text, see page 11, first paragraph.

A2) Regarding why we did not detect a statistically significant effect of colchicine on mean hop speeds in H2O cultures, we want to point out that not every experiment must always show patterns of detectable differences in exact agreement with every previous experiment. Our set-up was different than the experiment we referenced. Smith et al. (2003) used confocal microscopy and imaged vesicle motion at 2-s intervals. We applied TIRF microscopy and imaged at 20-ms time intervals. Therefore, velocity vectors for each frame were shorter in TIRF microscopy than in confocal microscopy. Since the camera projects a three-dimensional path of a moving object onto a two-dimensional plane, the component of vesicle velocity parallel to the substrate is not the full vesicle velocity vector. If only a small parallel component of an outward (from cell center towards cell-substrate contact zone) directed vesicle velocity vector is seen in our set-up, there is no reason to insist that the parallel component by itself be large enough to generate a statistically detectable difference. D2O treated cells appear to have larger vesicle velocities than H2O treated cells. The cells with larger overall velocities also have larger parallel components, and those larger parallel components show a statistically significant change with the application of colchicine. We don't need to insist on a statistically detectable change for the H2O group in order to be in accordance with previous research. To avoid this misunderstanding regarding the significance of the statistical significance test itself, we have now added this explanation to the manuscript body, see page 10, second paragraph.

A3) We agree that specific staining of the secretory granules would have helped in the interpretation of the data. We pointed this already out in the manuscript. Page 11, bottom of 1st paragraph:

> "However to fully investigate deuterium oxide's microtubule stabilization effect on secretory vesicle transport, future experiments that clearly identify secretory vesicles are warranted. For example, one can follow Smith et al. (2003) and transfect RBL-2H3 cells

with a green fluorescent protein-Fas ligand fusion protein (GFP-FasL) to study the transport of GFP-labeled secretory vesicles in more detail."

We looked into Referee 1's suggestion to use acridine orange as vesicle stain. It is true that this stain has been shown to be exocytosed with mast cell stimulation and that it can be found in low pH secretory granules. However according to Demo et al. (Cytometry 36:340, 1999), acidotropic dyes, such as acridine orange, have also been shown to accumulate in low pH cellular compartments, such as lysosomes and endosomes. Moreover, acridine orange interacts with DNA and RNA (see http://www.enzolifesciences.com/ENZ-52405/acridine-orange-ultra-pure/) and would therefore also label mast cell exosomes that contain RNA. Hence acridine orange would not be a specific enough label for mast cell granules and we believe that transfecting mast cells with GFP would be a better way to specifically stain for secretory granules. Hence, we made no changes within the manuscript in response to this reviewer's comment.

B) The term "within the resolution limit" needs some clarification.
We agree with Referee 1 that the vesicle cutoff size needs to be specified. The vesicle cutoff size corresponding to the resolution of the microscope was a Gaussian with variance of 200 nm, see page 6, second paragraph.

Finally, we are delighted to hear that we addressed all concerns raised by Reviewer 2. We hope that with the additional content revision that includes new data analysis our manuscript can now be accepted for publication in PeerJ.

Thank you for your consideration.

Sincerely, Kathrin Spendier
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