

Leiden, March 15th, 2021

Dear editors,

Herewith we respectfully resubmit our revised manuscript **“Flow cytometry and confocal imaging analysis for lowly Wnt expressing Axin2-mTurquoise2 reporter thymocytes”** by Jolanda J.D. de Roo, Brigitte A. E. Naber, Sandra. A. Vloemans, Edwin F. E. de Haas, Annelies M. A. van der Laan and Frank J.T. Staal for publication in the *Journal of Visualized Experiments*.

We would like to thank all four reviewers for their helpful comments and have tried to adapt the manuscript according to their indications although sometimes the comments were contradicting each other. Our manuscript aims to be a helpful protocol for the existing Axin2-mTurquoise2 canonical Wnt signaling reporter model for its suitability in low protein expression experiments, aiming at both the hematology community as well as the neurological community reader where therapeutic targets for the modulation of canonical Wnt signaling is emerging. Our protocol demonstrates the details of how to obtain a reliable fluorescent measurement in fragile thymocytes which are known to have a high apoptotic rate and relatively high background signal in the blue-green fluorochrome regions, as well as have a much-condensed cytoplasm which hinders localized fluorescent signal detection. Herewith we explain the highlighted changes that have been made per reviewer, including the editorial comments:

Editorial team

- We have improved on or followed all the comments from the editorial team.

Reviewer #1

- We have adjusted the major concern of reviewer #1 on the interpretation of the Wnt-on and Wnt-off states in the representative results section and side-by-side data in Figure 3. The wildtype Axin2-mTurquoise2 cells are not Wnt silent, they simply do not possess the reporter protein to visualize Wnt signaling.
- All minor concerns and specific comments have been improved by primarily more explanation in the protocol, representative results section or figure legends.

Reviewer #2

- We have answered all the mayor concerns and point by point comments to the best of our knowledge or fitting within the journal/ editorial team guidelines:
 - Spelling mistakes and less suitable word choices have been corrected.
 - The applicability of the protocol has been adapted or highlighted where possible without losing the aim of a relatively broad application. The other three reviewers were positive about the broad applicability of our model.
 - The specificity in the protocol has been changed where indicated. The steps where special care should be taken have been pointed out for the reader while still trying to respect the editorial team guidelines.
 - The quantitative aspects of the protocol have been addressed with additional testing and demonstration of negative controls. The background correction in the confocal data was reanalyzed and reevaluated with the imaging department and we have come to the conclusion that following the previously published Corrected Total Cell Fluorescence formula represent our data in the best manner. Negative/unstained controls had less background noise than in the triple-colored images, for which background correction in the triple-colored images seemed most fair. We have shown data of wildtype samples for the reader to see the level of background noise.
 - Concerns about the confocal laser settings have been carefully reevaluated and further optimized where possible. Otherwise, caution statements have been added to the manuscript for the reader.
 - Comments on the figures have been respected and changed.

Reviewer #3

- We have not followed the recommendation to include more therapeutic opportunities as this was a comment from another reviewer not to do so. We mention several neurological therapeutic opportunities in the introduction and hope to attract the interest of the neurological community sufficiently to see the applicability of our model/protocol for other non-defined neurological diseases.

Reviewer #4

- We have completed the representative results section as requested.

We hope your share our conviction that these data now warrant publication in *the Journal of Visualized Experiments*.

Sincerely, for the authors,

Jolanda J.D. de Roo, MSc
 PhD student in Immunology,
 Leiden University Medical Center