*10th September 2018,*

**Re : Resubmission of manuscript « Behavioural and Physiological analysis in a zebrafish model of epilepsy »**

**Bing Wu, Ph.D.**

**Review Editor**

**JoVE**

Dear Dr. Wu,

Thank you for your feedback and for the opportunity to revise our manuscript « **Behavioural and Physiological analysis in a zebrafish model of epilepsy »**. We have modified our manuscript according to the reviewers’ and editorial comments and we certainly hope that you will find our revised version suitable for publication in the Journal of Visualized Experiments.

Please find enclosed our letter of response to the editor’s and reviewers’ comments detailing the implemented modifications.

Thank you for you consideration.

Sincerely,

Sorana Ciura

Researcher at Imagine Institute

24 Blvd Montparnasse

75015 Paris France

Tel : 01 57 27 43 12

**Editorial comments:**

**Changes to be made by the Author(s) regarding the manuscript:**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

The manuscript has now been carefully proofread by all our co-authors.

**2. Please provide an email address for each author.**

We have now added the email adresses for each author.

**3. Please add a Summary section before the Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”**

We added the following description lines 19 to 20. « Here, we present a protocol for the development and the characterization of a zebrafish model of epilepsy resulting from the transient inhibition of the Depdc5 gene. »

**4. Line 31: Please define hpf.**

We spelled out the acronym hpf : « hours post fertilization ».

**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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: SYLGARD, Sigma Aldrich, Gene Tools, Parafilm, Instant Ocean, Grasshopper 2, FLIR, Viewpoint, Alomone Labs, Molecular Devices, RAW SIGNAL, etc.**

We have removed the commercial languages and used generic terms.

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

We eliminated the use of personal pronouns from the protocol section.

**7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.**

The imperative tense is now used throughout the protocol section.

**8. 1.1.5: Please describe how to prepare microinjection borosilicate glass needles.**

We have now included a description of the preparation of needles in section 1.1.5 (lines 140 to 145)

**9. 2.1.8, 2.2.12, 3.4: Software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.**

We explained the steps of softwares in section/lines 2.2.12/279-287.

**10. After you have made all the recommended changes to your protocol (listed above), 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.**

**11. 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. Please do not highlight any steps describing anesthetization and euthanasia.**

**12. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.**

We have highlighted the essential steps of the protocol to be vizualized.

**13. Please remove the embedded Figure 1 from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.**

We have separated the figure from the main text.

**14. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.**

We have revised our discussion and detailed the critical steps as well as some suggestions for troubleshooting (sections/lines 1.1.6./147-156 ; 1.3.5./196-200 ; 2.1.1./212-214 ; 2.1.5./224-226).

**15. References: References should be numbered in order of appearance. Please do not abbreviate journal titles.**

We have revised our reference annotations.

**16. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.**

Citations and references have been modified as indicated and the journal titles have been entirely spelled out.

**17. Table of Materials: Please combine all relevant materials in only one table. Please upload only the complete table.**

The table has been updated.

**Reviewers' comments:**

**Reviewer #1:**

**Title: Behavioural and Physiological analysis in a zebrafish model of epilepsy**

**General comments:**

**This study presents a method for generating a zebrafish depdc5 loss-of-function model and the protocol for behavioral phenotyping at 28 and 48 hours post fertilization (hpf). After a careful evaluation, the manuscript could be suitable for publication in Journal of Visualized Experiments (Jove), after minor revisions.**

**Specific comments:**

**1. The abstract and introduction sections are informative and describes clearly the purpose of this protocol.**

We thank the reviewer for this assessment.

**2. Protocol section: All animal research should comply with standard ethical guidelines. Considering that zebrafish embryos were used here to provide preliminary data (Fig 01). Were the protocols approved by the Institutional Animal Care and Use Committee, and are these in accordance with the standard ethical guidelines??**

We included the following text : « Experimental procedures were approved by the National and Institutional Ethical Committees. » (line 115).

**3. The concentration of AMO is determined empirically for each gene using a dose-response curve and represents a concentration where the AMO is effective in knocking down the gene without causing general toxicity, such as gross morphological defects. In this protocol what concentration was used? How could we replicate such data?**

We have now added to the manuscript the concentration used (0.4 mM, line 154).

**4. Using a video camera (Grasshoper2, FLIR) attached to a dissection microscope to record the spontaneous coiling activity. How many times are the embryos recorded?**

Groups of ten to twelve embryos per experiment were recorded one time, in a randomized manner as described. For this particular measurement, the experiment was repeated three times.

**5. The caption needs to be reviewed since the authors report two captions for the figure 01.**

We have now removed the embedded Figure 1 and its caption from the text.

**6. Discussion section: This section needs to be improved since only few references were provided. This point is of major concern in order to ensure a rationale for theoretical support based on previously published articles that explain the experimental procedure.**

We have now revised our discussion and included relevant references as suggested.

**Reviewer #2:**

**This manuscript reports a novel method for analyzing epilepsy in zebrafish larvae. The authors have presented a method for generating a zebrafish depdc5 loss-of-function model and the protocol for behavioral phenotyping at 28 and 48 hours post fertilization. The experimental protocol was clearly described. My main issues are four-fold:**

**1- Which time do morpholino fish display loss of function?**

The morpholino is effective as soon as it reaches the cytoplasm where it can contact the mRNA and constitute a steric hindrance to its translation.

**2- The authors must describe the mortality rate of subjects injected with morpholino. This is important for conducting an experiment with a reduced number of cohorts.**

We included the mortality rate in the text (section/line 2.1.5./224-226).

**3- Are transiently silenced subjects more susceptible to seizures when adults? Do the authors know about adult zebrafish, which have more complex convulsive behaviors than larvae?**

We did not test adult zebrafish. However, given the role of DEPDC5 in the mTOR pathway and in development, it is possible that adults are sensitized to seizure-inducing drugs.

**4- Please, describe the advantages and disadvantages of the morpholino model over chemically induced seizure models (ptz, kainic acid)?**

We added the following sentence in the discussion (lines 408-413) : « The AMO-mediated gene knock-down is a powerful technique, displaying advantages over chemically-induced seizure models, as it specifically targets the expression of a gene of interest, thus allowing the identification of the underlying pathogenic mechanisms triggered by a genetic mutation. Chemical inducers, which are nevertheless potent tools for drug screenings, can act through multiple cellular pathways that might not be always relevant to the genetic mutation under study. »

**Reviewer #3:**

**Manuscript Summary:**

**This paper discusses the behavioral and physiological analysis of a zebrafish model of epilepsy.**

**Major Concerns:**

**Major revisions are needed including the addition of results and discussion of positive and negative controls and rescue experiments and a power analysis to determine how many fish are needed per condition.**

Positive and negative controls, as well as recue experiments results have been published, see de Calbiac et al, Depdc5 knockdown causes mTOR-dependent motor hyperactivity in zebrafish. Annals of Clinical and Translational Neurology. doi : 10.1002/acn3.542 (2018). We have referenced this article in the text (reference 18). The power analysis (90%) indicates a sample size of 8 fish per condition.

**Minor Concerns:**

**Introduction:**

**Line 68 mentions treatment with rapamycin reversing the behavioral features in this model. These results are not included in the results or discussion. Please include or if not to be included, why not? Or has this data been published elsewhere and just a reference is needed?**

These data have been published and are now references in the text (reference 18 : de Calbiac et al, Depdc5 knockdown causes mTOR-dependent motor hyperactivity in zebrafish. Annals of Clinical and Translational Neurology. doi : 10.1002/acn3.542 (2018).)

**Line 71- please include a reference.**

References 14-18 have been included :

Teng, Y. et al. Knockdown of zebrafish lgi1a results in abnormal development, brain defects and a seizure-like behavioral phenotype. Human Molecular Genetics. doi: 10.1093/hmg/ddq364 (2010).

Baraban, S.C., Dinday, M.T., Hortopan, G.A. Drug screening in Scn1a zebrafish mutant identifies clemizole as a potential Dravet syndrome treatment. Nature Communications. doi: 10.1038/ncomms3410 (2013).

Suls, A. et al. De novo loss-of-function mutations in CHD2 cause a fever-sensitive myoclonic epileptic encephalopathy sharing features with dravet syndrome. American Journal of Human Genetics. doi: 10.1016/j.ajhg.2013.09.017 (2013).

Grone, B.P. et al. Epilepsy, behavioral abnormalities, and physiological comorbidities in syntaxin-binding protein 1 (STXBP1) mutant zebrafish. PLoS ONE. doi: 10.1371/journal.pone.0151148 (2016).

de Calbiac, H. et al. Depdc5 knockdown causes mTOR-dependent motor hyperactivity in zebrafish. Annals of Clinical and Translational Neurology. doi: 10.1002/acn3.542 (2018).

**Line 74- Please spell out AMO.**

We spelled out antisense morpholino oligonucleotides in the text.

**Protocol-**

**Lines 123-124- Please indicate 5' and 3' for each morpholino.**

We added the 5’ and 3’ directions in morpholino sequences.

**Line 131- Please double check that 0.6 g/L instant ocean is correct.**

We thank the reviewer for noticing this and we have now corrected the concentraton which is actually 0.06 g/L.

**Section 1.1.6- Please include a concentration range or starting point for the AMOs used.**

We added the following sentence in the text : « Typically, AMOs concentrations will be in a range of 0.2 mM to 1mM (0.4 mM was determined as the working solution in our case) » in the section/line 1.1.6./147-156.

**Line 172- Did you mean "top of needle"?**

Here we meant the sharp tip of the needle.

**Section 1.3.3- Please include how the volume of 2nL was calculated.**

We included the following sentence in the text : « You can calculate the injected volume by injecting the solution in a drop of mineral oil. Take a picture and measure the diameter of the injected fluid sphere. »

**Section 1.3.4- Is there a reason why you pass through the yolk sac to inject into the cells?**

We consistently observed less damages to the embryo using this strategy.

**Line 202- Could you use a 96-well plate instead of the plastic mesh grid in a petri dish?**

96-well plate wells are too large for imaging several embryos at high magnification. Also, we needed the embryos to stay at the exact same place. Both these aspects are critical for the ViewPoint analysis.

**Section 2.1.6- would the zebrabox work for recording the coiling activity?**

Yes, but we preferred using a higher magnification as available on a microscope.

**Line 216- "Repeat the experiment.."**

We corrected the sentence.

**Sections 2.1.7 & 2.2.10- Please specify how many embryos are needed for each condition with power analysis.**

For a power of 90%, 8 fish are needed for each condition.

**Section 2.1.8- Was total activity analyzed? What were the parameters for inactive, small, large activity?**

Total activity was analysed using the Quantitation module of the Zebralab. The parameters for freezing and burst were 10 and 50, respectively (the inactive, small and large parameters only apply to the Tracking module). This information was added to the relevant section.

**Section 2.2.12- Why was zebralab not used as in section 2.1.8?**

At 48 hours post fertilization (hpf) spontaneous swimming is limited, so we quantified the robust escape response to a tactile stimulus, which cannot be reproduced in the zebrabox.

**Line 268- Please subscript where appropriate.**

We have now corrected the formulae.

**Representative Results-**

**Line 358- "Depdc5 KD shows a higher occurrence of spontaneous events"; were statistics run on this data? If so, was it significantly higher? Why aren't the 28 and 48 hpf data included? Why aren't the + and - control data included? Why aren't the rescue experiments included? Discussion-Need to add discussion on the rescue experiments.**

These data have been published and are now references in the text (reference 18 : de Calbiac et al, Depdc5 knockdown causes mTOR-dependent motor hyperactivity in zebrafish. Annals of Clinical and Translational Neurology. doi : 10.1002/acn3.542 (2018)).

**Acknowledgements-**

**Font seems to change throughout this section.**

We corrected the font.

**Figures-**

**Figure 1- How old are the fish for this figure?**

We have now mentioned the age of the fish (4-6 dpf) in the text (lines 107, 368 and 431).