Dear Editor,

Thank you very much for handling our manuscript.

According to the comments from the editors and reviewers, we revised the manuscript. The followings are our responses to the comments.

We hope that we addressed all the comments, and the manuscript is now suitable for publication in Journal of Visualized Experiments.

Sincerely yours,
Kazuhide Asakawa PhD

Editorial comments:

Changes to be made by the Author(s):

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

Answer: The revised manuscript has been checked by the English proofreading service.

2. Please revise the following lines to avoid previously published work: 58-59, 306-309, 482-484.

Answer: We revised these sentences (Lines 55, 326, 485).

3. Please ensure that abbreviations are defined at first usage.

Answer: We have checked the definitions of abbreviations.

4. 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. Please sort the Materials Table alphabetically by the name of the material.

Answer: We made the corrections accordingly.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Answer: We tried to be as detailed as possible in the revised manuscript. To put the protocol steps in a chronological order we made an independent section for LED calibration as in "3. Preparation of LED for blue light illumination" (Line 184). In the originally submitted manuscript, this step was placed between the imaging the steps, but this step needs to be done before imaging starts.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Answer: We kept each step no more than 4 sentences.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly.
Answer: We used imperative tense throughout.

8. Please add more details to your protocol steps:
Step 1.1.1: Please clatrify what is meant by BAC containing the zebrafish.
Answer: It is a zebrafish BAC clone containing the mnr2b locus. We made the correction (Line 93).

Step 1.1.2: Please mention how the BAC transformation was performed.
Answer: BAC transformation was done as in (Warming, 2005). The procedures of BAC transformation by (Warming, 2005) is reliable but contain many steps, which we think are too long to be included in this manuscript. We would like readers to refer the methods described in (Warming, 2005).

Step 1.2.1/1.3.1: Please mention how the plasmid construction was done or cite published articles here.
Answer: We cited reference for these plasmids (Lines 112 and 141).

Step 1.2.2: Please provide details of the PCR reaction, the different parameters, and components. Also please mention the primers used throughout.
Answer: We provided the detailes of PCR reactions in lines 103 and 118

Step 1.2.3: Please specify all gel running conditions throughout V/cm, temperature, etc.
Answer: We specified the electrophoresis conditions (Line 127).

Step 2.2: Please specify what is meant by the one-cell stage.
Answer: This is a conventional way of describing the zebrafish embryo before the first cell division (line 155).

Step 2.4: Please mention how the crosses are set. Is there an equal number of variants for each type?

Answer: We mentioned how the crosses are set (line 164).

Step 3.5: Is the size of the syringe needle critical here? If so, please mention it.

Answer: The size of the needle is not critical.

Step 4.2: What spacer is used here?

We used five slice glasses stacked, and mentioned to it in the text (line 242).

9. In the software, please ensure that all button clicks and user inputs are provided throughout. Also, please ensure that the button clicks are bolded.

Answer: We made sure about these points.

10. Please spell out the journal titles in the References.

Answer: The journal titles are spelled out.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Kazuhide Asakawa et al. describe an optogenetic TDP-43 reverse translational model of ALS, from cloning and transgenesis through to data processing.

Major Concerns:

No major concerns

We would like to thank the Reviewer #1 for the positive evaluation of the manuscript.

Minor Concerns:

A few points warrant clarification or amendment

1.1.2. Note unclear: should the BAC be thawed the same day for higher success rate, or the day before?

Answer: Timing of the BAC purification from the E. coli cells affects the outcome. We corrected the sentences as shown in Line 98

2.2. line 158, remove "the" from "the adulthood"

Answer: The sentence was corrected as suggested (Line 159).

4.1. Replace "iPad/iPhone" with "tablet/phone" (Nanoleaf is also available for android devices)

Answer: The sentence was corrected as suggested (Line 187).

Reviewer #2:

Manuscript Summary:

The authors explain how to visualize the ontogenetic phase transition of certain protein in zebrafish larvae. Although there are some minor issues to improve the video and the manuscript, their explanation is easy to understand for other researchers and worth publishing in JoVE.

Major Concerns:

None.

We would like to thank the Reviewer #2 for the positive comments and valuable

suggestions.

Minor Concerns:
Protocol:
Line 151. Please describe the origin or how to prepare the Tol2 transposase mRNA.
Answer: We cited a paper describing how to prepare the mRNA (Line 152).

Line 184. Please describe the composition of E3 medium.
Answer: The composition of E3 medium was described in the line 178.

Line 229. Please describe the company name and version info for Fiji/ImagJ.
Answer: We described the version info and cited a paper that reported Fiji (Line 254).

Line 261. Need period at the end of the sentence.
Answer: Corrected as suggested (Line 284).

Line 256. Need company name and version info for GraphPad Prism.
Answer: We added the information (Line 279).

Representative results:
To reduce redundancy, please remove the details for how to perform the experiments, or moved to protocol section, if necessary.
Answer: Some of the descriptions of confocal imaging was removed or moved to the PROTOCOL section.

Figure 2B. Please explain how to image (or decide the same position during microscopic observation) the same location of 48 and 72 hpf larvae.
Answer: We think that the sentence “the cloaca on the ventral side of the fish was included in the ROI as a reference, helping to identify and compare the spinal segments (levels 16-17) across the time points” (in Line 228) should suffice for that purpose.

Discussion:
I prefer the last paragraph.
Answer: Thank you very much.

Line 465. "beound" would be "beyond".

Answer: Corrected as suggested (Line 468).

Reviewer #3:

Manuscript Summary:

Asakawa and colleagues have given a protocol describing how they generated a novel zebrafish model of ALS using a bacterial artificial chromosome-based genetic method to deliver an optogenetic variant of TDP-43. They have also included a protocol on imaging and on image analysis.

The protocols are reasonably clear- particularly the image acquisition and processing.

Major Concerns:

No major concerns identified

We are very grateful for a number of comments and suggestions from Reviewer #3's, which greatly helped us improve this manuscript. .

Minor Concerns:

Manuscript could be improved by the following.

General: give the full name of genes the first time of mention.

Answer: We added the full name of TDP-43 in its first mention (Line 20).

Is there a limit to the detection/analysis of the zebrafish (ie larval timeframe only) due to pigment formation?

Answer: We thank the Reviewer#3 for pointing this out. We add N-Phenylthiourea to inhibit the melanogenesis, which we described in the revised manuscript (Line 179).

Remind the reader that the zebrafish are double transgenics thoughout the paper

Answer: We described fish as double-transgenic fish whenever they are.

Could discuss more how the method could be expanded (different promoter) to other disease indications with a similar protein aggregation pathology

Answer: We mentioned to a possible application of this system to TDP-43 proteinopathies in general with relevant promoters (Line 485).

Intro

Line 80: explain in intro how to selectively target this cell type.

Answer: We mentioned that we used BAC transgenesis (Line 75).

Protocol

Line 97: explain mnr2b here or during introduction

Answer: We explained mnr2b in Introduction (Line 75)

Line 98: Query: missing a step- to innoculate the e.coli with the BAC (although perhaps this is covered in Warming et al)

Answer: The detailed steps are described in Warming et al 2005. Every detail is important for BAC transgenesis, so we would like to avoid its incomplete description in this protocol.

Line 103: what is meant by preparation? Is it the purification step in 1.1.1?

Answer: We corrected preparation to purification (Line 98).

Line 107: Cat # for the iTol2-amp cassette- or instructions on how someone can obtain it

Answer: We cited Suster et al 2011, in which the iTol2-amp cassette was developed (Line 321).

Line 110: is there a specific way to identify e.coli cell clones carrying the BAC and the iTol2-amp cassette?

Answer: Integration of the iTol2-amp cassette is confirmed by PCR. We provided the details in Line 103

Line 114: typo "flanked"

Answer: the typo was corrected (Line 114).

Line 115: hsp70l (650 bp). Give a ref for this promoter sequence.

Answer: We added a reference (Line 116).

Line 152: ref/cat # for tol2 transposase mRNA

Answer: We added a reference (Line 152).

Line 156: give the timeframe for RFP screening (e.g. 2 hours, 8 hours, 7 days).

Answer: We added the timeframe (Line 158).

Line 178: The timeline for imaging the larvae is not given. I assume initial image at 48

hpf and the 24 hours +/- blue light and then reimage... but this is not clear in the protocol. Were the zebrafish dechorionated (my larvae are still within the chorion at 48 hpf).
Answer: We added the timeline in Protocol 4 and 5.

Line 182: 42C... this suggests that all of this protocol was done at 42C (which I assume not) perhaps clarify.
Answer: We modified the sentence Line 211.

Line 196: How long does it take the agarose to solidify and how long does the imaging take- for the novice... indicative timing... how long can the zebrafish tolerate in low melt agarose before it affects welfare. I know this is later in the paper- but helpful to give during the protocol.
Answer: We added the information in 4.5, 4.7. and 4.9..

Section 4: Be clear that this follows on directly from step 3.
How are controls treated? Here it suggests that the zebrafish are treated for 24 hours in blue light... are the controls also in light conditions through the night (potential for effect on circadian rhythm).
Answer: We mentioned to non-illuminated control in 5.2 (Line 245). We haven't detected overt effect of circadian rhythm on opTDP-43 at this early stage.

Line 219: Describe how the larva is removed from the low-melt agarose. Confirm 7.5 ml (seems a lot for a 6-well plate).
Answer: It is described in 4.9. We have used 7.5 ml of E3 buffer per well.

Line 224: give time (larva is now 72 hpf.. for example)
Answer: We added "72 hpf" in 4.4 (Line 249).

Section 6.1: Explain where the background subtraction is done.
Answer: Subtraction step was mentioned in 7.5.(Line 298)

Line 321: give attrition rate for this method.
Answer: We are sorry that we did not quite understand what the Reviewer #3 meant by attrition rate. The neurons do not migrate away from the ROIs during and between imaging, but sometimes it is difficult to confirm identities of cells in the images of different time points when they become too close to each other. We do not know how

often this happens, but we added one sentence in “Find the cells that are identifiable in both images at 48 hpf and 72 hpf, based on the relative positions of the cell bodies of the spinal motor neurons” in Line 268.

Line 355: Give an indication: how many single neurons need to be evaluated per experiment?
Answer: We have analyzed 65 single cells from three independent animals in total in (Asakawa, 2020) and found no statistically significant difference between opTDP-43h/EGFP-TDP-43z ratio between 48 hpf and 72 hpf, which is mentioned in Line 382.

Figure 1: Good.
Answer: Thank you very much.

Figure 2: I think this could benefit from the inclusion of a timeline describing the experiment as a part A- ie take current part A and change to a timeline. Give age of larva too.
Scale bar: one for zebrafish should not be 1 mm- please correct. Zebrafish are not this length at 72 hpf.
Answer: According to the suggestions, we revised figure 2A.

Figure 3 (and discussion/result): how is data average. Comparing 1 cell to 1 cell allows for the introduction of bias- how did the researcher select the cells- what bias mitigation was done here/should the reader do.
Answer: We selected cells that are identifiable in both images at 48 hpf and 72 hpf, based on the relative positions of the cell bodies, as mentioned in Line 268. We discussed about the average from 65 cells from three independent animals described in Asakawa 2020 in line 382.

Line 424: Unclear what the author means by "dark" conditions... assumed that zebrafish either under blue light or in normal light/dark cycling conditions... add more detail. In addition- unsure if zebrafish would have "normal" motor behaviour (line 425) if in the dark. Consider impact of dark on locomotion or at least comment on it.
Answer: We would like to thank the reviewer for this comment. As written in the revised manuscript in 5.2. (Line 245), dark condition means a condition where light is shut by aluminum foil cover. We agree with Reviewer #3 that behaviors of fish raised without light is not the same as those of fish raised under normal light/dark cycle. Thus, we

avoided using “normal”, and rather described the phenotypes of Tg[mnr2b-hs:opTDP-43h] fish that suggested that the cytotoxicity of opTDP-43h was minimal (Line 429).

Line 428: provide a reference.
Answer: We provided a reference (Line 434).

Line 449: could mnr2b bac inserts incorporate at > 1 locus?
Answer: We think that it is possible but quite rare. Most of our mnr2b- BAC F1s gave ~50 % reporter-positive in F2s, suggesting that one locus carry the BAC insert.

Line 465: typo
Answer: The typo was corrected (Line 468).

Line 478: this method shows from 48 to 72 hours- not 120 hpf.
Answer: We corrected the sentence and cited (Asakawa 2020) for longer illumination (Line 480).

Line 479: blue light may impact visual function.
Answer: We mentioned to an effect on vision in Line 482.