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TITLE:

Optogenetic Phase Transition of TDP-43 in Spinal Motor Neurons of Zebrafish Larvae

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neurodegenerative disease, ALS, optoDroplet system, Cryptochrome 2, TDP-43, zebrafish, spinal motor neuron, *mnr2b,* BAC transgenesis

SUMMARY:

We describe a protocol to induce phase transition of TAR DNA-binding protein 43 (TDP-43) by light in the spinal motor neurons using zebrafish as a model.

ABSTRACT:

Abnormal protein aggregation and selective neuronal vulnerability are two major hallmarks of neurodegenerative diseases. Causal relationships between these features may be interrogated by controlling the phase transition of a disease-associated protein in a vulnerable cell type, although this experimental approach has been limited so far. Here, we describe a protocol to induce phase transition of the RNA/DNA-binding protein TDP-43 in spinal motor neurons of zebrafish larvae for modeling cytoplasmic aggregation of TDP-43 occurring in degenerating motor neurons in amyotrophic lateral sclerosis (ALS). We describe a bacterial artificial chromosome (BAC)-based genetic method to deliver an optogenetic TDP-43 variant selectively to spinal motor neurons of zebrafish. The high translucency of zebrafish larvae allows for the phase transition of the optogenetic TDP-43 in the spinal motor neurons by a simple external illumination using a light-emitting diode (LED) against unrestrained fish. We also present a basic workflow of live imaging of the zebrafish spinal motor neurons and image analysis with freely available Fiji/ImageJ software to characterize responses of the optogenetic TDP-43 to the light illumination. This protocol enables the characterization of TDP-43 phase transition and aggregate formation in an ALS-vulnerable cellular environment, which should facilitate an investigation of its cellular and behavioral consequences.

INTRODUCTION:

Ribonucleoprotein (RNP) granules control a myriad of cellular activities in the nucleus and cytoplasm by assembling membrane-less partitions via liquid–liquid phase separation (LLPS), a phenomenon in which a homogeneous fluid demixes into two distinct liquid phases[1,2]. The dysregulated LLPS of RNA-binding proteins that normally function as RNP granule components promote abnormal phase transition, leading to protein aggregation. This process has been implicated in neurodevelopmental and neurodegenerative diseases[3-5]. The precise evaluation of a causal relationship between aberrant LLPS of RNA-binding proteins and disease pathogenesis is crucial for determining whether and how LLPS can be exploited as an effective therapeutic target. LLPS of RNA-binding proteins is relatively easy to study *in vitro* and in unicellular models but is difficult in multicellular organisms, especially in vertebrates. A critical requirement for analyzing such LLPS in individual cells within a tissue environment is to stably express a probe for the imaging and manipulation of LLPS in a disease-vulnerable cell type of interest.

Amyotrophic lateral sclerosis (ALS) is an ultimately fatal neurological disorder in which motor neurons of the brain and spinal cord are selectively and progressively lost due to degeneration. To date, mutations in more than 25 genes have been associated with the heritable (or familial) form of ALS, which accounts for 5%–10% of total ALS cases, and some of these ALS-causing genes encode RNA-binding proteins consisting of RNPs, such as hnRNPA1, TDP-43, and FUS[6,7]. Moreover, the sporadic form of ALS, which accounts for 90%–95% of total ALS cases, is characterized by the cytoplasmic aggregation of TDP-43 deposited in degenerating motor neurons. A major characteristic of these ALS-associated RNA-binding proteins is their intrinsically disordered regions (IDRs) or low-complexity domains that lack ordered three-dimensional structures and mediate weak protein-protein interactions with many different proteins that drive LLPS[7,8]. The fact that ALS-causing mutations often occur in the IDRs has led to the idea that aberrant LLPS and phase transition of these ALS-related proteins may underlie ALS pathogenesis[9,10].

Recently, the optoDroplet method, a Cryptochrome 2-based optogenetic technique that allows the modulation of protein-protein interactions by light, was developed to induce phase transition of proteins with IDRs[11]. As this technique has been extended successfully to TDP-43, it has begun to uncover the mechanisms underlying pathological phase transition of TDP-43 and its associated cytotoxicity[12-15]. In this protocol, we outline a genetic method to deliver an optogenetic TDP-43 to ALS-vulnerable cell types, namely, spinal motor neurons in zebrafish using the BAC for the *mnr2b*/*mnx2b* gene encoding a homeodomain protein for motor neuron specification[16,17]. The high translucency of zebrafish larvae allows for simple, noninvasive light stimulation of the optogenetic TDP-43 that triggers its phase transition in the spinal motor neurons. We also present a basic workflow for the live imaging of the zebrafish spinal motor neurons and image analysis using the freely available Fiji/ImageJ software to characterize the responses of the optogenetic TDP-43 to the light stimulation. These methods allow for an investigation of TDP-43 phase transition in an ALS-vulnerable cellular environment and should help to explore its pathological consequences at cellular and behavioral levels.

PROTOCOL:

All fish work was conducted in accordance with the Guide for the Care and Use of Laboratory

Animals of the Institutional Animal Care and Use Committee (approval identification number 24-2) of the National Institute of Genetics (Japan), which has an Animal Welfare Assurance on file (assurance number A5561-01) at the Office of Laboratory Animal Welfare of the National Institutes of Health (NIH, USA).

1. Construction of BACs for expression of optogenetic TDP-43 gene from the *mnr2b* promoter

1.1.1. Purchase a zebrafish BAC clone containing the zebrafish *mnr2b* locus (CH211-172N16, BACPAC Genomics). Purify the BAC DNA from a 5 mL overnight LB culture of DH10B *Escherichia coli* (*E. coli*) harboring CH211-172N16 as described in Warming et al.[18].

1.1.2. Transform CH211-172N16 into SW102 *E. coli* cells by electroporation, as described in Warming et al.[18].

NOTE: Purification of the BAC DNA from the *E. coli* on the same day of electroporation usually gives a higher success rate of BAC transformation[18]. Otherwise, the purified BAC DNA is kept at −20 ˚C until use.

1.1.3. Introduce the iTol2-amp cassette for *Tol2* transposon-mediated BAC transgenesis[19] into the backbone of CH211-172N16 by electroporation, as described in Asakawa et al.[20].

1.1.3.1. Make a glycerol stock of the *E. coli* cell clones carrying CH211-172N16 with the iTol2-amp cassette integration (CH211-172N16-iTol2A) after confirming the homologous recombination-mediated integration by polymerase chain reaction (PCR) (Ex Taq) using the primer pair Tol2-L-out (5'-AAA GTA TCT GGC TAG AAT CTT ACT TGA-3') and pTARBAC-13371r (5'-TAG CGG CCG CAA ATT TAT TA-3') and the following conditions: a denaturation step at 98 °C for 1 min, followed by 25 cycles of denaturation at 95 °C for 10 s, primer annealing at 55 °C for 15 s, and primer extension at 72 °C for 1 min, amplifying a PCR product of 354 base pairs (bp).

1.2.1. Construct a plasmid carrying the expression cassette for the human wild-type TDP-43/*TARDBP* (TDP-43h) that is tagged with mRFP1 and CRY2olig at the N- and C-termini, respectively (hereafter, opTDP-43h)[14].

NOTE: The opTDP-43h fragment should be flanked with the zebrafish *hsp70l* gene promoter sequence (650 bp) and polyadenylation (polyA) signal sequence, followed by a kanamycin resistance gene (*hsp70l*p-opTDP-43h-polyA-Kan)[14].

1.2.2. Amplify the *hsp70l*-opTDP-43h-polyA-Kan cassette with primers that anneal *hsp70l* and Kan and contain 45 bp sequences of the upstream and downstream of the initiator codons of the *mnr2b* gene by PCR (GXL DNA Polymerase) using the primer pair mnr2b-hspGFF-Forward (5'-tat cag cgc aat tac ctg caa ctc taa aca caa caa aag tgt tgc aGA ATT CAC TGG AGG CTT CCA GAA C-3') and Km-r (5'-ggt tct tca gct aaa agg gcg tcg atc ctg aag ttc ttt gac ttt tcc atC AAT TCA GAA GAA CTC GTC AAG AA-3') with the following conditions: a denaturation step at 98 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 10 s, primer annealing at 55 °C for 15 s, and

primer extension at 68 °C for 30 s, amplifying a PCR product of ~5.7 bp.

1.2.3. Separate the PCR products by agarose gel electrophoresis (100 V) and purify the *hsp70l*p-opTDP-43h-polyA-Kan DNA band with a DNA column. Adjust the concentration of the purified *hsp70l*p-opTDP-43h-polyA-Kan cassette to 50 ng/μL in Tris-EDTA buffer (TE) containing 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

1.2.4. Introduce the *hsp70l*p-opTDP-43h-polyA-Kan cassette into CH211-172N16-iTol2A by electroporation as described in Warming et al.[18] and select ampicillin- and kanamycin-resistant transformants on LB agar plates. CH211-172N16-iTol2A carrying the *hsp70l*p-opTDP-43h-polyA-Kan cassette is designated as mnr2b-hs:opTDP-43h.

1.2.5. Purify mnr2b-hs:opTDP-43h using a BAC purification kit and dissolve it at 250 ng/μL in TE after phenol/chloroform extraction.

1.3.1. Construct another plasmid carrying the zebrafish wild-type *tardbp* that is tagged with enhanced green fluorescent protein (EGFP) at its N-terminus (EGFP-TDP-43z) instead of opTDP-43h but is otherwise identical to the *hsp70l*-opTDP-43h-polyA-Kan construct (hsp70l-EGFP-TDP-43z-polyA-Kan)[14]. Use EGFP-TDP-43z as an internal control for the light stimulation of opTDP-43h.

1.3.2 Construct CH211-172N16-iTol2A harboring the *hsp70l*p-EGFP-TDP-43z-polyA-Kan cassette in the *mnr2b* locus as described in 1.2.1–1.2.5. CH211-172N16-iTol2A carrying the *hsp70l*p-EGFP-TDP-43z-polyA-Kan cassette is designated as mnr2b-hs:EGFP-TDP-43z.

2. *Tol2* transposon-mediated BAC transgenesis in zebrafish

2.1. Prepare the injection solution containing 40 mM KCl, phenol red (10% v/v), 25 ng/μL of the mnr2b-hs:opTDP-43h DNA, and 25 ng/μL *Tol2* transposase mRNA[19].

2.2. Inject 1 nL of the injection solution (a droplet with a diameter of approximately 123 μm calibrated in mineral oil) into the cytosol of wild-type zebrafish embryos at the one-cell stage. Screen the injected fish for the formation of red fluorescent protein (RFP)-positive aggregates in various embryonic tissues, including spinal motor neurons, under a fluorescence stereomicroscope at 2–3 days post-fertilization (dpf). Raise the RFP-positive fish to adulthood.

2.3. Inject the mnr2b-hs:EGFP-TDP-43z DNA as described in 2.1 and 2.2. Raise EGFP-positive fish to adulthood.

2.4. After a few months, put sexually matured injected fish and wild-type fish in pairs in standard 2 L mating cages to obtain F1 offspring. Screen F1 fish at 3 dpf for RFP (opTDP-43h) or EGFP (EGFP-TDP-43z) fluorescence in the spinal motor column using an epifluorescence microscope equipped with a Plan-Neofluar 5x/0.15 objective lens. Typically, one founder fish is identified from 10−20 injected fish (the germline transmission rate is 5%−10%).

2.5. Isolate and compare multiple Tg[mnr2b-hs:opTDP-43h] and Tg[mnr2b-hs:EGFP-TDP-43z] inserts from different founder fish, as the intensity, but not the pattern, of opTDP-43h or EGFP-TDP-43z expression may vary between founder fish due to chromosomal position effects.

2.6. Cross Tg[mnr2b-hs:opTDP-43h] and Tg[mnr2b-hs:EGFP-TDP-43z] fish lines to obtain offspring containing Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish in a Mendelian ratio.

2.7. Raise the fish in a plastic dish containing 30 mL of E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% Methylene Blue) and add 0.003% (w/v) N-phenylthiourea at 8–10 h post-fertilization (hpf) to inhibit melanogenesis.

2.8. Cover the plastic dish with aluminum foil after 30 hpf.

3. Preparation of LED for blue light illumination

3.1. Turn on an LED panel by using the associated application installed on a tablet/phone. Put the probe of a spectrometer into an empty well of a 6 well dish and adjust the LED light to the wavelength peaking at ~456 nm through the application. Place the optical sensor of an optical power meter in the empty well and adjust the power of the LED light (~0.69 mW/cm^2). The LED light setting can be saved and is retrievable in the application.

3.2 Introduce the dish/LED panel setting to the incubator at 28 °C. Finish this step before imaging of fish starts at 48 hpf.

4. Imaging of zebrafish larvae expressing optogenetic TDP-43

4.1. Select Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish at least before 47 hpf, based on RFP (opTDP-43h) or EGFP (EGFP-TDP-43z) fluorescence in the spinal motor column using the epifluorescence microscope set-up described above.

4.2. Dechorionate the Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish.

4.3. Preheat 1% low melting temperature agarose containing 250 µg/mL of ethyl 3-aminobenzoate methanesulfonate salt at 42 °C.

4.4. Briefly anesthetize Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43] double-transgenic fish at 48 hpf in E3 buffer containing the same concentration of Tricane.

4.5. Put a drop of the preheated 1% low melting temperature agarose on the glass base dish at the room temperature. The diameter of the dome-shaped agarose drop on the glass dish is 8–10 mm.

4.6. Using a Pasteur pipette, add the anesthetized fish to the low melting temperature agarose on the glass base dish, and then mix by pipetting a few times. Minimize the amount of the E3 buffer added to the agarose along with the fish.

4.7. Maintain the fish on its side by using a syringe needle during the solidification of agarose (typically ~1 min) to ensure that the spinal cord is in an appropriate horizontal position. After the solidification, put a couple of drops of E3 buffer onto the dome-shaped agarose-mounted fish.

4.8. Acquire serial confocal z-sections of the spinal cord by scanning with a confocal microscope equipped with a 20x water immersion objective lens with the numerical aperture 1.00, using a scan speed of 4.0 µs per pixel (12 bits per pixel), a step size of 1.0 µm per slice for the objective, and a combination of excitation/emission wavelengths: Channel 1) 473/510 nm for EGFP and Channel 2) 559/583 nm for mRFP1.

NOTE: The cloaca on the ventral side of the fish is included in the regions of interest (ROI) as a reference, which helps to identify and compare the spinal segments (levels 16–17) across the time points.

4.9. Remove the fish from the agarose by carefully cracking the agarose with a syringe needle as soon as the imaging is complete. Keep the amount of time the fish is embedded in the agarose as short as possible, although the agarose embedding for <30 min does not affect the viability of the fish.

5. Light stimulation of opTDP-43h-expressing fish by field illumination of a blue light-emitting diode (LED) light

5.1. Add 7.5 mL of E3 buffer to the well and place the imaged Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish into the well. Place the six-well dish on the LED panel by keeping the dish and LED panel 5 mm apart with a spacer (for example, with five slide glasses stacked).

5.2. Turn on the blue LED light. Keep some of the Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish in a separate six-well dish covered with aluminum foil when unilluminated control fish are necessary (i.e., in dark conditions)[14].

5.3. After the illumination (e.g., for 24 h at 72 hpf in **Figure 3**), image the spinal cord of the illuminated fish by repeating the steps 4.3 – 4.9.

6. Visualization of cytoplasmic relocation of optogenetic TDP-43 in the spinal motor neurons

6.1. Open the image file in ImageJ/Fiji [21] (Version: 2.1.0/1.53c), an open-source Java image processing program developed by NIH Image, which can be downloaded from https://imagej.net/Fiji/Downloads.

6.2. Use the Z scrollbar to move through the focal planes. Create a maximum intensity projection of multiple slices by clicking **Image | Stacks | Z project | Max Intensity** and setting Start slice and Stop slice that cover the hemisegments of the spinal motor column.

6.3. Split the multichannel image into two single-channel images by clicking **Image | Color | Split Channels.**

6.4. Enhance the EGFP-TDP-43z signal to ensure that cytoplasmic EGFP-TDP-43z is visible clearly by clicking **Image | Adjust | Brightness/Contrast** and adjusting **Minimum**

6.5. Open the ROI manager by clicking **Analyze | Tools | ROI manager**. Find the cells that are identifiable in both images at 48 hpf and 72 hpf, based on the relative positions of the cell bodies. Set the ROIs by outlining the contours of cell bodies of single spinal motor neurons visualized by the EGFP-TDP-43z signal using **Freehand selections**, and then clicking **Add[t]** in the ROI manager.

6.6. Set a major axis of the soma by drawing a straight line using **Straight** and clicking **Analyze | Plot Profile** for EGFP-TDP-43z (C1) and opTDP-43h (C2) images at 48 and 72 hpf.

6.7. Normalize the Plot Profile by dividing the values by the greatest value for each EGFP-TDP-43z and opTDP-43h signal. Plot the normalized values with x–y coordinates, where x and y represent the major axis of the soma and relative fluorescent intensities, respectively, using XY graph in a statistical software.

7. Ratiometric comparison between opTDP-43h and EGFP-TDP-43z signals using ImageJ/Fiji

7.1. Open the image file in ImageJ/Fiji and set ROIs for single *mnr2b*-positive cells using a maximum intensity projection image of EGFP-TDP-43z as in 6.1-6.5. Add a ROI outside the ventral spinal cord (e.g., notochord) to represent the background signal (background ROI).

7.2. For each EGFP-TDP-43z and opTDP-43 image, create a projection of multiple slices by clicking **Image | Stacks | Z project | Sum Slices** and set Start slice and Stop slice that cover the hemispinal motor column.

7.3. Open the ROI manager created with the maximum intensity projection image. Display the ROIs on the Sum Slices image by selecting the image window for EGFP-TDP-43z and then clicking **Show All** in the ROI manager. Click **Measure** in the ROI manager to obtain mean values for each ROI.

7.4. Acquire mean values for opTDP-43h following the same procedure provided in 7.3.

7.5. For each ROI, after subtracting the mean for the background ROI, divide the subtracted mean for opTDP-43h by the subtracted mean for EGFP-TDP-43z to obtain the ratiometric value. The ratiometric values can be compared between different time points and presented using Column graph in Prism software.

REPRESENTATIVE RESULTS:

Live imaging of optogenetic and non-optogenetic TDP-43 proteins in the *mnr2b*+ spinal motor neurons of zebrafish larvae

To induce TDP-43 phase transition in the spinal motor neurons in zebrafish, a human TDP-43h that is tagged with mRFP1 and CRY2olig[22] at the N- and C-termini, respectively, was constructed and designated as opTDP-43h[14] (**Figure 1A**). The opTDP-43h gene fragment was introduced into a BAC containing the *mnr2b* locus (**Figure 1B**). The resulting BAC, designated as mnr2b-hs:opTDP-43h, was introduced into the zebrafish genome by *Tol2* transposon-mediated BAC transgenesis[19]. To monitor the localization of non-optogenetic TDP-43 in the spinal motor neurons, a zebrafish TDP-43 encoded by *tardbp* gene was tagged with EGFP at the N-terminus (**Figure 1A**) and the EGFP-TDP-43z gene fragment was introduced into the mnr2b BAC, similar to opTDP-43h (**Figure 1B**). The resulting BAC, designated as mnr2b-hs:EGFP-TDP-43z, was introduced into the zebrafish genome by *Tol2* transposon-mediated BAC transgenesis. The fish injected with each BAC construct was crossed with a wild-type fish and the resulting F1 fish were screened on day 3 for red (Tg[mnr2b-hs:opTDP-43h]) or green (Tg[mnr2b-hs:EGFP-TDP-43z]) fluorescence in the ventral spinal cord. The isolated red or green fluorescence-positive F1 fish were designated as Tg[mnr2b-hs:opTDP-43h] or Tg[mnr2b-hs:EGFP-TDP-43z], respectively.

Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish at 48 hpf were anesthetized and embedded in a low melting agarose on their side; after agarose solidification, they were covered with E3 buffer to allow observations of the lateral side of the spinal cord from above. Confocal laser scanning microscopy was performed with a 20x water immersion objective lens and a combination of excitation/emission wavelengths: 473/510 nm for EGFP and 559/583 nm for mRFP1. The scanned fish was immediately and carefully taken from the agarose, transferred into E3 buffer in a six-well plate, and illuminated by a blue LED light by placing the plate on a LED panel at 28 °C (**Figure 2A,B**). After a 24-h illumination, the fish was anesthetized and embedded again in agarose before being scanned with a confocal microscopy using the same imaging conditions, except that the ROI was adjusted to include the corresponding spinal cord region imaged at 48 hpf (**Figure 2B**). The whole hemispinal cord of 4–5 contiguous spinal segments was imaged during the microscopy session (typically 20–30 min), generating z-series images containing 20–40 slices depending on the horizontalness of the longitudinal axis of the spinal cord of the mounted fish relative to the confocal scanning plane.

Visualization of cytoplasmic relocation of optogenetic TDP-43 in spinal motor neurons

To visualize the cytoplasmic relocation of opTDP-43h in single spinal motor neurons, the z-series images (typically 20–40 slices) acquired at 48 and 72 hpf were opened with Fiji and separated into each EGFP-TDP-43z and opTDP-43h channel. Max intensity projection images of EGFP-TDP-43z were created for images at 48 and 72 hpf and a single isolated spinal neuron identifiable in both images at 48 and 72 hpf was selected (**Figure 2B, arrows**). Spinal motor neurons with cell bodies located on the dorsal side of the motor column were considered suitable for

measurements of the cell body shape because of their sparse distribution patterns.

After adjusting the intensity of the EGFP signal in EGFP-TDP-43z images, ROIs covering the somas of the motor neurons were set by tracing the edge of the EGFP signal at 48 and 72 hpf (**Figure 2C**). The fluorescent intensity along the major axis of the soma was measured for the EGFP-TDP-43z and opTDP-43h signals at 48 and 72 hpf (**Figure 2C, right**). At 48 hpf, the patterns of opTDP-43h and EGFP-TDP-43z signals largely overlapped each other.

In contrast, at 72 hpf, the peak of the opTDP-43h signal was found in the region where the EGFP-TDP-43z signal was low, namely in the cytoplasm, indicating the cytoplasmic relocation of opTDP-43h. The light-dependent cytoplasmic opTDP-43h relocation is initiated largely independently of non-optogenetic TDP-43[14]. In this assay, Max intensity projection images were used to estimate the position of the nucleus/cytoplasm boundary at the expense of quantitative measurements.

Ratiometric comparison of the fluorescence intensity of optogenetic and non-optogenetic TDP-43 in the spinal motor neurons

To evaluate the effects of the blue light stimulation on the fluctuating opTDP-43h protein levels, opTDP-43h signals in the cell body were measured before and after the light stimulation with reference to the EGFP-TDP-43z signal. The z-series images, including the spinal hemisegment, were opened with Fiji. To set ROIs covering single spinal motor neurons, Max intensity projection images of the EGFP-TDP-43z signal were created for 48 and 72 hpf, and single isolated spinal neurons that were identifiable in both 48 and 72 hpf images were selected (**Figure 3A**). Using freehand selections, ROIs were drawn and registered in a ROI manager for each of the 48 and 72 hpf images (**Figure 3A**). Max intensity projection of the EGFP-TDP-43z signal was considered suitable to set the ROIs because of its high contrast.

To quantify the opTDP-43h and EGFP-TDP-43z signals, Sum slices projection images for each channel of the same z-series images were produced for 48 and 72 hpf (**Figure 3A**). Using the registered ROI sets, the fluorescent intensities of opTDP-43h and EGFP-TDP-43z were measured for each time point. Relative amounts of opTDP-43h to EGFP-TDP-43z (RFP/GFP values) were calculated for each cell and time point by dividing the RFP value by the GFP value (**Figure 3B**). Of the four *mnr2b*-positive cells that were investigated, cell #1 notably displayed a saturated EGFP-TDP-43z signal (**Figure 3A**). Cells with saturated fluorescent signals should be excluded from the data sets when conducting quantitative analyses. Cells #2–#4 displayed decreasing trends of relative opTDP-43h levels to EGFP-TDP-43z after blue light illumination (**Figure 3B**), although a larger-scale analysis previously demonstrated that the decrease in relative opTDP-43h levels to EGFP-TDP-43z was not statistically significant (65 cells from three independent animals)[14].

FIGURE LEGENDS:

Figure 1: Construction of BAC DNA using the *mnr2b* locus. (**A**) Structures of the expression cassettes for opTDP-43h and EGFP-TDP-43z. (**B**) The zebrafish genomic region carried by the CH211-172N16 BAC DNA. The PCR-amplified expression cassettes for opTDP-43h and EGFP-TDP-

43z are inserted downstream of the 5'-untranslated region (UTR) of the *mnr2b* (red arrow) in the first exon of *mnr2b* by homologous recombination. The bars indicate 500 (A) and 10k (B) bp.

Figure 2: Live imaging of opTDP-43h before and after light stimulation. (**A**) A scheme of the light stimulation of opTDP-43h expressed in the spinal motor neurons of unrestrained Tg[mnr2b-hs:opTDP-43h] Tg[mnr2b-hs:EGFP-TDP-43z] double-transgenic fish through a field illumination of blue LED light from 48 to 72 hpf. (**B**) Max intensity projection images of the z-series confocal images of the ventral spinal cord before (48 hpf) and after (72 hpf) the light stimulation. The dashed lines demarcate the ventral limit of the spinal cord. Figures are adapted from Asakawa et al.[14] (**C**) Cytoplasmic mislocalization of opTDP-43 after the 24-h blue light illumination. The contours of the soma of an mnr2b-positive cell (red arrows in B) observed at 48 and 72 hpf are shown in magenta. The major axes of the somas were shown with light blue. The normalized fluorescent intensity along the major axes was plotted. The asterisk indicates a cluster of strong opTDP-43 signals that do not display a strong EGFP-TDP-43z overlapping signal, indicating cytoplasmic opTDP-43h mislocalization. The bars indicate 1 mm (A), 20 μm (B), and 4 μm (C).

Figure 3: Ratiometric comparisons of opTDP-43h and EGFP-TDP-43z before and after light stimulation. (**A**) ROIs covering the somas of four single mnr2b-positive cells at 48 and 72 hpf were drawn based on the EGFP-TDP-43z signal and are shown in magenta. The rectangular ROIs (bg) were used to subtract the background signal (background ROI). Figures are adapted from Asakawa et al.[14] (**B**) The relative intensities of opTDP-43h to EGFP-TDP-43z were plotted for each cell at each time point as the RFP/GFP ratio. Cell #1, indicated by the asterisk, was not suitable for the ratiometric comparison because its EGFP-TDP-43z signal was saturated and its RFP/GFP value was overestimated. The bar indicates 10 μm.

DISCUSSION:

The *mnr2b*-BAC-mediated expression of opTDP-43h and EGFP-TDP-43z in zebrafish provides a unique opportunity for live imaging of TDP-43 phase transition in the spinal motor neurons. The optical transparency of body tissues of zebrafish larvae allows for the simple and noninvasive optogenetic stimulation of opTDP-43h. Comparisons between single spinal motor neurons over time demonstrated that the light-dependent oligomerization of opTDP-43h causes its cytoplasmic clustering, which is reminiscent of ALS pathology.

One of the critical parameters that define the phase behavior of an intrinsically disordered protein is intracellular concentration. A high level of opTDP-43h expression could potentially lead to light-independent opTDP-43h oligomerization, phase transition, and aggregation. Thus, the induction of a stable, nontoxic level of opTDP-43h expression in the spinal motor neurons is key to the successful evaluation of the physiological and pathological consequences of opTDP-43 phase transition on the spinal motor neurons. The *mnr2b*-BAC-mediated expression of opTDP-43 h appears to be suitable for this purpose because Tg[mnr2b-hs:opTDP-43h] fish display predominantly nuclear opTDP-43, they are capable of free swimming with an inflated swim bladder and they maintain full viability even after being raised under the continuous dark conditions from 1–5 dpf. In zebrafish, a conventional approach used to exogenously express a gene of interest is mRNA injection at the one-cell stage. However, a high dose of the TDP-43

protein translated from the injected mRNA all at once causes early developmental defects [14] that preclude analyses of later differentiating spinal motor neurons. However, lowering the amount of injected mRNA to a tolerable level fails to supply sufficient opTDP-43h for optogenetic modulation in the spinal motor neurons by the time of its differentiation, indicating that mRNA injection is not a suitable method by which to deliver opTDP-43h to the spinal motor neurons of zebrafish. Another possible approach used to express opTDP-43h in the spinal motor neurons is injecting, at the one-cell stage, a plasmid or BAC DNA harboring the opTDP-43h construct under the control of a promoter that is active in the spinal motor neuron. Although the injection of mnr2b-hs:opTDP-43h BAC DNA can direct the expression of opTDP-43h in the spinal motor neurons, the number of opTDP-43h-positive motor neurons is low, and in such numerically restricted opTDP-43h-positive cells, the expression level is usually high, often associated with light-independent opTDP-43h aggregation. These observations collectively suggest that transgenic expression is an irreplaceable strategy by which to stably express opTDP-43h in the spinal motor neurons at a nontoxic level. Notably, although the *mnr2b*-BAC labels almost all spinal motor neurons in zebrafish[20,23], the expression levels of opTDP-43h could vary among individual *mnr2b*-positive cells. This variation may be partly due to the intrinsic expression pattern of *mnr2b* associated with its regulatory role in motor neuron differentiation. Another potential cause for the variegated expression may result from a chromosomal position effect on the *mnr2b*-BAC inserts. Whatever the cause, the varied opTDP-43 expression levels among *mnr2b*-positive cells could cause variation in cytotoxicity associated with light-dependent opTDP-43h phase transition.

In zebrafish larvae, the somas of spinal motor neurons are densely aligned in the ventral spinal cord, and the somal cytoplasm has a much lower volume than that of the axonal cytoplasm. This anatomical feature limits quantitative analyses of cytoplasmic opTDP-43h in the spinal motor neurons: opTDP-43h is only quantitatively measurable in the nucleus and somal cytoplasm, but not in the entire cell of motor neurons. Quantitative imaging of a protein in the whole cell of spinal motor neurons, including the soma and the peripheral nerve terminal, remains a challenging task. Despite the restrictions on quantitative assays, the opTDP-43h/EGFP-TDP-43z ratio in the soma was shown to be elevated by the ALS-causing mutation in the IDR (A315T)[14]. Therefore, the present method is applicable to evaluating the effects of the TDP-43 mutation on protein stability associated with phase transition in the soma of spinal motor neurons.

In principle, the *mnr2b*-BAC approach can be extended to modulate the LLPS and evaluate the cytotoxicity of other ALS-related RNP proteins with IDRs beyond TDP-43. Several protocol steps may need to be adjusted to obtain an optimal expression level of such optogenetic probes in the spinal motor neurons. First, in the Tg[mnr2b-hs:opTDP-43h] transgenic construct, the *hsp70l* promoter was used as a basal promoter to boost opTDP-43h expression driven by *mnr2b* enhancers. The *hsp70l* promoter may be removed from the BAC construct if the expression level of a protein of interest is so high that it causes light-independent ectopic phase transition. Second, opTDP-43h is equipped with CRY2olig tag, which is a photolyase homology (PHR) domain carrying a point mutation enhancing the clustering capacity on blue light illumination[22]. The wild-type protein $CRY2_{PHR}$ may be used as a light-dependent oligomerization tag if there is a need to attenuate light-dependent clustering activity. Finally, to more faithfully recapitulate ALS

pathology in zebrafish, it is desirable to establish an illumination protocol where fish physiology is minimally affected by the field illumination of blue light during the juvenile and adult stages. The present method uses a continuous blue light illumination from 48 to 72 hpf (for 24 h), and the illumination duration can be extended until 120 hpf (for 72 h) without losing fish viability[14], although light-responsive physiological functions, such as vision, may be affected. By developing a protocol for intermittent light illumination, much longer-term light stimulation may become possible, which may in turn facilitate the development of opTDP-43h into more mature pathological aggregates. To achieve this, other optogenetic probes for modulating protein–protein interactions using different light wavelengths that are less physiologically disturbing[24] may also be worth investigating further. Combinations of such improved optogenetic TDP-43 probes, appropriate promoters targeting disease-vulnerable cell types, and illumination protocols with minimal effects on physiological functions would open avenues for faithfully modeling the pathologies of TDP-43 proteinopathies not only in the spinal cord but also in the brain.

ACKNOWLEDGMENTS:

This work was supported by SERIKA FUND (KA), KAKENHI Grant numbers JP19K06933 (KA) and JP20H05345 (KA).

DISCLOSURES:

KA and KK are the inventors of the intellectual property described in this manuscript and provisional patents have been submitted by the National Institute of Genetics.

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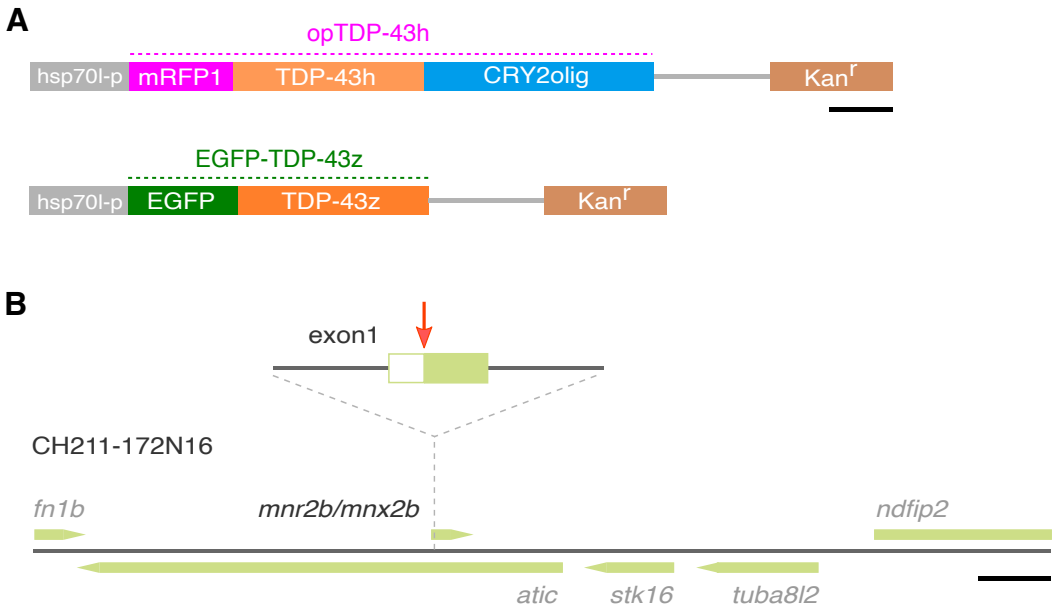
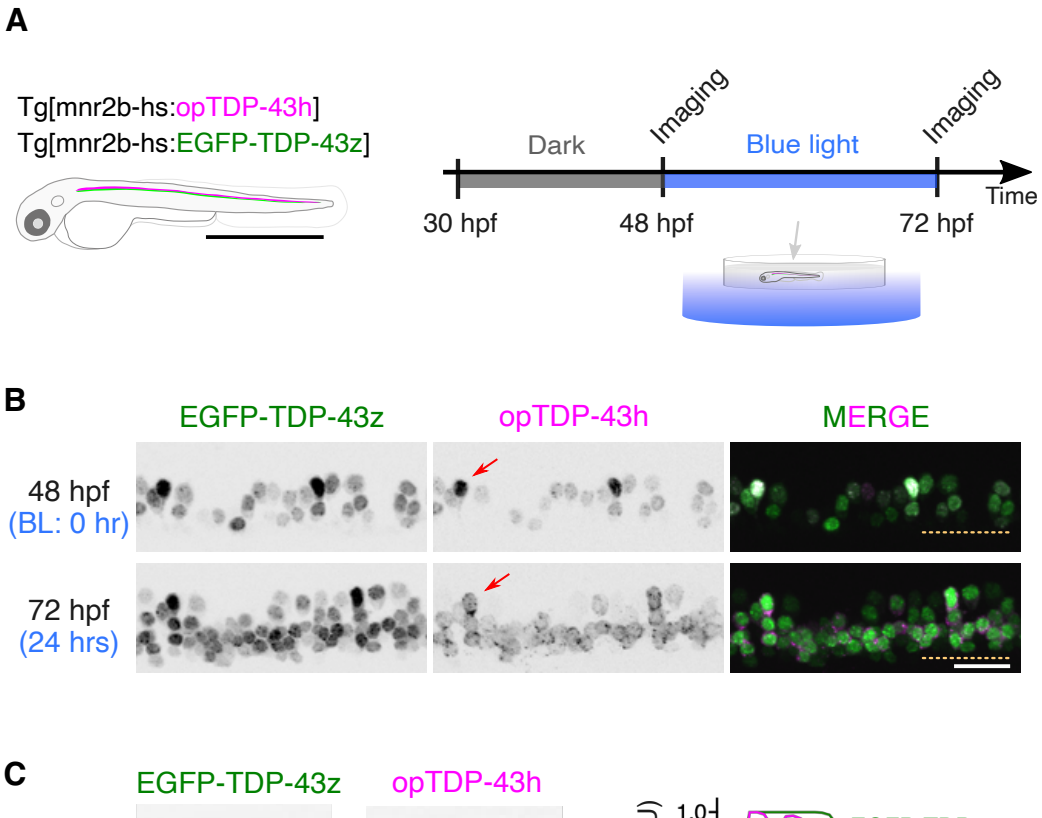
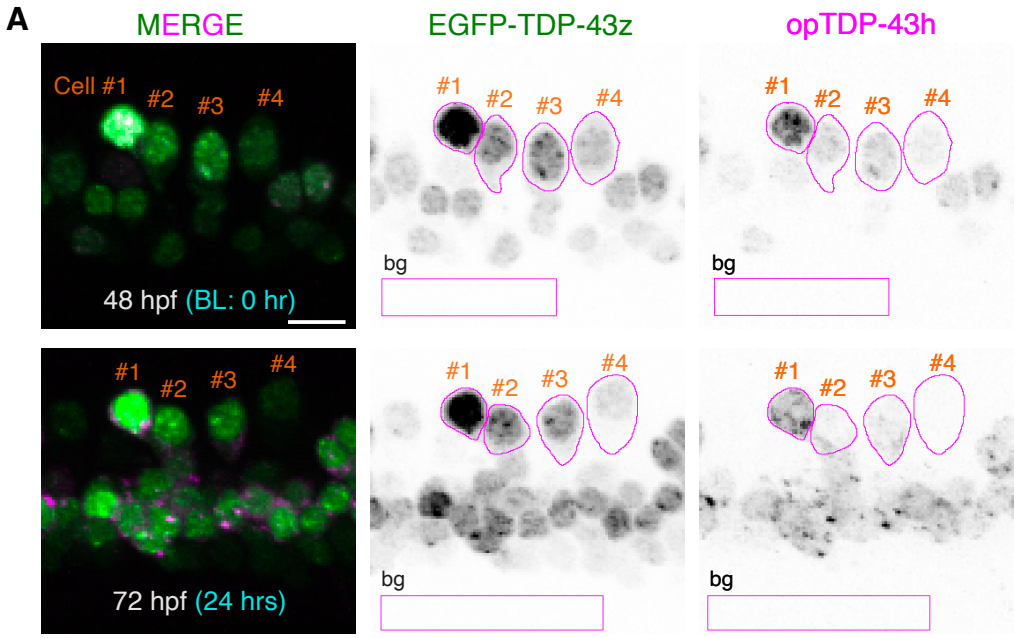
Asakawa et al. Figure 1

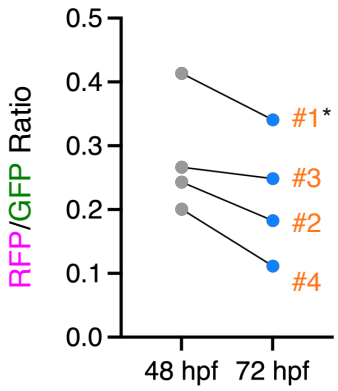


Asakawa et al. Figure 2



Asakawa et al. Figure 3





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.