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## Behavioral and Physiological Analysis in a Zebrafish Model of Epilepsy
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1 **TITLE:**
2 Behavioral and Physiological Analysis in a Zebrafish Model of Epilepsy
3

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22 **KEYWORDS:**
23 zebrafish, epilepsy, anti-epileptic drug discovery, epileptogenesis, genetics, in vivo
24 electrophysiology, neuronal circuits, *DEPDC5*, mTOR, translational neuroscience
25

26 **SUMMARY:**
27 Here, we present a protocol for the development and the characterization of a zebrafish
28 model of epilepsy resulting from the transient inhibition of the *DEPDC5* gene.
29

30 **ABSTRACT:**
31 Epilepsy represents one of the most common neurological disorders, affecting an estimated
32 50 million people worldwide. Recent advances in genetic research have uncovered a large
33 spectrum of genes implicated in various forms of epilepsy, highlighting the heterogeneous
34 nature of this disorder. Appropriate animal models are essential for investigating the
35 pathological mechanisms triggered by genetic mutations implicated in epilepsy and for
36 developing specialized, targeted therapies. In recent years, zebrafish has emerged as a
37 valuable vertebrate organism for modeling epilepsies, with the use of both genetic
38 manipulation and exposure to known epileptogenic drugs, such as pentylenetetrazole (PTZ),
39 to identify novel anti-epileptic therapeutics. Deleterious mutations in the mTOR regulator
40 *DEPDC5* have been associated with various forms of focal epilepsies and knock-down of the
41 zebrafish orthologue causes hyperactivity associated with spontaneous seizure-like
42 episodes, as well as enhanced electrographic activity and characteristic turn wheel
43 swimming. Here, we described the method involved in generating the *DEPDC5* loss-of-
44 function model and illustrate the protocol for assessing motor activity at 28 and 48 h post
45 fertilization (hpf), as well as a method for recording field activity in the zebrafish optic
46 tectum. An illustration of the effect of the epileptogenic drug PTZ on neuronal activity over
47 time is also provided.

## INTRODUCTION

Due to its small size, oviparous development and transparency at early stages of development, zebrafish has emerged as a valuable vertebrate organism for modeling human diseases as diverse as cardiovascular, cancer or neurological disorders[1,2]. Zebrafish combines the advantages of a vertebrate, including the high conservation of organ architecture and genetic code, with the small size and ease of genetic manipulation of simpler model organisms, therefore facilitating both fundamental studies and translational applications. In particular, its amenability to high-throughput automated screening of behavior and fluorescent markers of cellular processes has made zebrafish a particularly attractive model for epilepsy research. This has been demonstrated by a high increase in the last decade of the number of publications featuring chemically-induced and/or genetic models of epilepsy[3–5] and, more recently, reports of promising therapeutics obtained from chemical screens in these models[6–8].

*DEPDC5* is a member of the GATOR1 complex, a negative regulator of mTOR signaling[9]. Mutations in the *DEPDC5* gene have been first discovered in 2013 in probands suffering from autosomal dominant focal epilepsies[10,11], and have since been reported in a number of clinical conditions associated with focal epileptic manifestations and focal cortical dysplasia[12]. The large majority of reported mutations are predicted to cause the loss-of-function of the gene[12], and this was formally demonstrated for a number of *DEPDC5* mutated transcripts which are targeted by nonsense mediated mRNA decay[12,13]. In agreement, knock-down of the gene orthologue in zebrafish using antisense morpholino oligonucleotides (AMOs) results in a number of features that are common to epileptic models in this organism, including hyperactivity, turn wheel-like swimming, spontaneous seizures and enhanced neuronal activity[14–18]. Interestingly, treatment with rapamycin, an inhibitor of mTOR signaling, reversed the behavioral features of this model[18], supporting the hypothesis that *DEPDC5* loss-of-function can trigger epilepsy due to a misregulation of the mTOR pathway[9,19].

Transient knock-down of gene expression in vivo using antisense oligonucleotides carrying the morpholino modification has been an invaluable tool for studying the role of specific genes, on par with si/shRNA-based techniques. Recently, AMO-based strategies have also found clinical applications, with a first AMO therapy receiving the FDA approval for the treatment of Duchenne muscular atrophy in 2016[20]. While it was reported that in zebrafish the phenotype of acute AMO-based gene knock-down does not always correlate with the constitutive knock-out models[21], this can be due at least in some instances to compensatory mechanisms engendered by constitutive genetic modifications[22]. However, the issue of specificity of the AMO-induced phenotype is an undisputable concern that has to be diligently addressed in studies using this technology[23]. In order to ensure the specificity of the AMO-based knock-down phenotype, several key controls are necessary. These include a dose-response curve that allows the selection of the lowest dose of AMO effective for gene knock-down, avoiding overall toxicity due to the introduction of an excess of genetic material. The use of a *Mismatch* AMO that does not target any particular region in the genome is also required for establishing an appropriate dose and in identifying a specific phenotype. A second AMO which targets a different region of the same gene, such as a splice-blocking AMO, is necessary to confirm that the phenotype is due to the knock-down

95  of the target gene. Rescue of the knock-down phenotype with the cDNA of the gene, either
96  the human orthologue or a codon-modified version of the zebrafish gene that cannot be
97  targeted by the AMO, provides a strong argument in favor of the phenotype specificity. Lack
98  of rescue with the same cDNA containing loss-of-function mutations (such as the
99  introduction of an early stop codons) is a further proof in this direction.
100
101  Here, we present a method for generating a zebrafish *DEPDC5* loss-of-function model and
102  the protocol for behavioral phenotyping at 28 and 48 h post fertilization (hpf). At 28 hpf,
103  *DEPDC5* loss-of-function causes overall hyperactivity, as evidenced by enhanced coiling and
104  twitching movements of the embryos within the chorion. An automated motion detection
105  system can be used at this stage to quantify the overall activity per embryo. At 48 hpf,
106  zebrafish exhibit stereotyped escape swimming in response to touch. In zebrafish with
107  downregulated expression of *DEPDC5*, the swimming trajectory is significantly more
108  tortuous than in controls, the fish exhibiting a "cork-screw" or "turn-wheel" like pattern,
109  similar to other reported epilepsy models in this organism[3,4]. Electrophysiological recordings
110  were obtained in the optic tectum in zebrafish larvae between 4-6 days post fertilization
111  (dpf) and show a baseline increase in neuronal activity in the *DEPDC5* knock-down animals.
112  The advantage of this model is that it presents several phenotypic features at different time
113  points, which can be useful in monitoring and assessing the efficacy of drug therapies during
114  development.
115
116  **PROTOCOL**
117  Experimental procedures were approved by the National and Institutional Ethical
118  Committees.
119
120  **1. Transient Knock-Down of *DEPDC5* Gene in Zebrafish Embryo**
121
122  **1.1. Preparation of tools:**
123
124  1.1.1. Prepare silicon elastomer-coated injection Petri dishes: Mix the base and curing agent
125  of the kit (see **Table of Materials**) in a 10:1 ratio. Fill a 35 mm Petri dish halfway with the
126  mixture. Wait for the silicon to harden before using it (this can take several days).
127
128  1.1.2. Prepare 1.2 mmol/L stock solutions of antisense morpholino oligonucleotides (AMO;
129  see **Table of Materials**). Add 250 µL of sterile water to 300 nmole lyophilized AMO to obtain
130  a 1.2 mmol/L stock solution. For complete dissolution, heat the vials for 5 min at 65 °C.
131  Vortex briefly. Seal the tube cap with a plastic film (see **Table of Materials**).
132
133  1.1.3. For the control rescue experiments, prepare an expression plasmid containing the
134  human cDNA of *DEPDC5* (see **Table of Materials**) cloned in the pCS2 backbone or a similar
135  zebrafish-compatible expression plasmid. As a negative control, a mutation causing an early
136  stop codon (p.Arg487*) was introduced in the cDNA.
137
138  1.1.4. Prepare embryo water: 0.06 g/L aquarium salt (see **Table of Materials**) in reverse
139  osmosis water + 0.5 mg/L methylene blue.
140

141  1.1.5. The day of the injection, prepare microinjection borosilicate glass needles using a
142  puller (see **Table of Materials**). Establish appropriate temperature settings on the needle
143  puller. Use a 10 cm long, 1/0.5 OD/ID mm borosilicate glass capillary to generate two ~5 cm
144  capillaries with thin tips with a length of approximately 1 cm.

145

146  1.1.6. If the tip of the needles is very fine, preventing the ejection of solution, break the very
147  end of the tapered tip using forceps under a microscope.

148

149  1.1.7. Just prior to injection, prepare the working solutions of AMOs. Always prepare fresh
150  solution to ensure the reproducibility of the results. Heat the AMO stock vials at 65 °C for 5
151  min. Prepare a 5 μL injection sample containing Fast Green dye (0.02% final concentration,
152  see **Table of Materials**) and the AMO diluted at the working concentration in water.

153

154  1.1.8. Determine the working concentration of the AMO empirically for each gene using a
155  dose response curve. The working concentration represents a concentration where the
156  AMO is effective in knocking down the gene without causing general toxicity, such as gross
157  morphological defects. Typically, AMO working concentrations will be in a range of 0.2
158  mmol/L to 1 mmol/L (0.4 mmol/L was determined as the effective concentration for this
159  study[18]). Inject the control Mismatch morpholino at the same concentration as the effective
160  AMO.

161

162  1.1.9. Vortex the tubes and centrifuge briefly to bring the droplets to the bottom of the
163  tubes.

164

165  1.1.10. For rescue experiments, prepare a 5 μL injection sample with the AMO diluted at the
166  working concentration and the cDNA expression plasmid diluted in water to a final
167  concentration to be determined empirically. For the expression of *DEPDC5* and the negative
168  control plasmid, 100 ng/μL was effective for phenotypic rescue.

169

170  **1.2. Embryo preparation:**

171

172  1.2.1. The day prior to microinjection, set up the zebrafish mating tanks. The morning of the
173  injection, remove the dividers to enable spawning. Collect the eggs in 100 mm Petri dishes
174  filled with embryo water using a fine sieve. Inject within 20-30 min from collection, while
175  the eggs are at the one cell stage.

176

177  1.2.2. Pick 60-80 eggs with a plastic Pasteur pipette and arrange them in the silicon-coated
178  Petri dish for injection. The silicon surface will prevent the eggs from sliding during the
179  injections. Remove most of the embryo water, leaving just enough to cover the eggs
180  halfway.

181

182  **1.3. Microinjections:**

183

184  1.3.1. Fill a glass needle with injection solution. Place the needle vertically in one of the
185  tubes containing the injection solution, ensuring that the bottom end of the needle is
186  touching the solution. Wait several minutes until the colored injection solution rises by
187  capillarity and is visible at the tip of the needle.

1.3.2. Mount the filled needle on the injecting handle of the microinjector (see **Table of Materials**).

1.3.3. Turn on the air compressor and adjust the pressure setting to generate an injection volume of ~2 nL.

1.3.4. To calculate the volume of the injected solution, place a drop of mineral oil on a microtome slide. Inject the dye-containing solution using the set pressure and time parameters. Measure the diameter of the injected fluid sphere and calculate total volume using the formula Volume=$4/3*\pi*(d/2)^3$, where d=the measured diameter of the injected bolus.

1.3.5. Using a dissecting binocular microscope with a 4X magnification, inject the eggs at the single cell stage by passing through the chorion and the yolk, and projecting the solution directly within the cell.

1.3.6. Collect the injected embryos in a 100 mm Petri dish with embryo water, label the dish and incubate them at 28 °C.

1.3.7. Ensure that the incubator temperature is stable over time, as embryos development rate is thermosensitive. For example, the growth would be accelerated at higher temperatures and the developmental stage is critical for properly assessing the phenotype[24].

1.3.8. Check the quality of the eggs 6-8 h after injection and remove dead and unfertilized embryos using a plastic Pasteur pipette.

1.3.9. The next morning, count and remove dead embryos in each dish with a plastic Pasteur pipette.

**2. Behavior Analysis**

**2.1. Global activity analysis at 28 hpf:**

2.1.1. Conduct the test the afternoon of the day after microinjection (28 hpf), ensuring that the time of day at which the test is conducted is consistent over experiments to perform valid statistical analysis as embryos development is very rapid.

2.1.2. Fill a 35 mm dish (test dish) with embryo water and allow it to warm up in the incubator (28 °C) for at least 15 min prior to starting the test.

2.1.3. Place a plastic mesh grid (1.2x1.2 mm) cut to size, on the bottom of the test dish.

2.1.4. Have another experimenter randomize the order of testing of the embryos and mask the names of the conditions to be tested.

235  2.1.5. Make sure that the mortality rate does not change among conditions and compared
236  to non-injected embryos to ensure the specificity of the phenotype. The percent of dead
237  embryos in all conditions shall not exceed 10-13%[18].
238
239  2.1.6. Place 10-12 embryos still within their chorion on the plastic mesh using a plastic
240  Pasteur pipette. Fill the test dish with enough embryo water to keep the embryos
241  submerged but not floating. If needed, move the embryos with care using a plastic tip to
242  position them on the grid.
243
244  2.1.7. Using a video camera (see **Table of Materials**) attached to a dissection microscope,
245  record the spontaneous coiling activity for a defined length of time (10-20 min long videos
246  are usually sufficient to obtain representative samples of activity bursts for the
247  quantification)
248
249  2.1.8. Return the embryos to their respective dish and put them back in the incubator.
250  Repeat the experiment with as many embryos as needed for each condition (as determined
251  by a 90% power analysis).
252
253  2.1.9. To analyze total spontaneous movement, use a ZebraLab system (see **Table of**
254  **Materials**). Using the activity quantification module, upload the recorded video and design
255  the tracking arenas around each embryo as appropriate. Set the freeze and burst threshold
256  to 10 and 50, respectively.
257
258  2.1.10. Run the automated video analysis, which quantifies total activity inside each of the
259  defined arenas, then recover the data set as a spreadsheet and perform the analysis using a
260  data analysis software.
261
262  **2.2. Touch-Evoked-Escape-Response (TEER) at 48 hpf:**
263
264  2.2.1. Conduct the test in the morning two days after the injection (48 h after the
265  fertilization).
266
267  2.2.2 At least 2 h prior to testing, dechorionate the embryos using fine forceps. Ensure that
268  the time of day for the dechorionation and the behavior test is consistent over experiments.
269
270  2.2.3. Fill a 130 mm dish (test dish) with embryo water and allow it to warm up in the
271  incubator (28 °C) for at least 15 min prior to starting the test.
272
273  2.2.4. Count and remove dead and morphologically deformed larvae. Record the numbers
274  for each condition.
275
276  2.2.5. Have another experimenter randomize the order and codify the names of the
277  conditions to be tested.
278
279  2.2.6. Mount the camera (see **Table of Materials**) over the test dish making sure that the
280  entire test dish is within the field of view. Placing a ruler within the field of view provides an
281  internal calibration for distance.

282

2.2.7. With a plastic Pasteur pipette, put an embryo in the center of the test dish and begin the recording using an acquisition rate of 30 fps.

2.2.8. With a fine plastic tip, touch slightly the tail of the embryo with a flicking motion.

2.2.9. Stop the recording when the larva has terminated its movement.

2.2.10. Remove the embryo from the test dish and place it in a new dish filled with embryo water. Repeat the test with as many embryos as needed for each condition (as determined by a 90% power analysis).

2.2.11. Return the embryos to their original dish and put them back in the incubator.

2.2.12. To analyze the parameters of the swimming behavior, load the recorded video to ImageJ analysis software. Download and install the Manual Tracking plugin of ImageJ (see **Table of Materials**). Launch the plugin by choosing **Tools | Plugin | Manual Tracking** in the menu.

2.2.13. In the dialog window, introduce the calibrated scale of the image. Including a ruler in the camera field facilitates the conversion of cm to pixels.

2.2.14. Select **Add track** and start the trajectory tracing by clicking on the image of the zebrafish larva in the first frame. The frames advance automatically with each point that is added to the trace.

2.2.15. Continue tracing the movement until the end of the swimming episode.

2.2.16. Select **End track** on tracking window, retrieve the X-Y coordinates and calculate total distance, velocity and turning angle.

**3. Electrophysiological Analysis**

**3.1. Reagent and tool preparation:**

3.1.1 Prepare 1% agarose in embryo water (see section 1.1.4). Aliquot the liquid agarose in microcentrifuge tubes and keep these on a heating block at 42 °C to prevent the agarose from hardening.

3.1.2. Prepare the recording solution (in mmol/L): NaCl 134, KCl 2.9, $CaCl_2$ 2.1, $MgCl_2$ 1.2, glucose 10, HEPES 10, pH 7.8.

3.1.3. Pull borosilicate glass micropipettes with a tip opening of 1.5-2 μm (5-6 $Mm^2 \cdot kg \cdot s^{-3} \cdot A^{-2}$ resistance) unpolished.

**3.2. Preparation of zebrafish larvae for electrophysiology:**

3.2.1. Place the fish in a glass-bottom Petri dish (see **Table of Materials**) and remove excess extracellular media in order to ensure the fish is brought as close to the cover slip as possible.

3.2.2. Using a plastic Pasteur pipette, add warm liquid agarose on and around the larva. Use just enough agarose to cover the fish. While the agarose hardens, use fine forceps to orient the fish in a straight position, ventral side down, in the center of the dish.

3.2.3. Add 2 mL of the recording solution containing 10 µM Pancuronium bromide (see **Table of Materials**) to block neuromuscular transmission. The addition of the paralyzer is necessary to eliminate artefacts due to small movements during the recordings.

**3.3. Electrophysiological recording**

3.3.1. Fill the micropipette with recording solution.

3.3.2. With the patch clamp amplifier (see **Table of Materials**) in voltage clamp configuration, measure electrode resistance in bath to confirm its correct value.

3.3.3. Using a 20X objective, position the head of the larva in the central field of view and lower the micropipette to reach the recording position in the brain, within the optic tectum.

3.3.4. Switch the patch clamp amplifier to current clamp and fix the holding current to 0 mA.

3.3.5. Using a low-pass filter of 1 kHz, an acquisition rate of 1 kHz and a digital gain of 10, record spontaneous activity for 60 min for determining baseline activity levels.

3.3.6. After 1 h of baseline recording, add 200 µL pentylenetetrazol (PTZ, see **Table of Materials**) solution 300 mmol/L to the bath for a final concentration of 20 mmol/L PTZ.

3.3.7. Record the neuronal activity in PTZ for another 120 min.

**3.4. Depolarization event determination**

3.4.1. Field recording events have very slow dynamics (frequencies of interest are in the range of 0.005-0.2 $s^{-1}$). Therefore, filter the signal with a low pass (Butterworth 5th order LPF at 100 $s^{-1}$) in order to avoid aliasing. Subsample the recorded voltage data from the acquisition frame rate (in this case, 1 $ks^{-1}$) down to 250 $s^{-1}$ (RAW SIGNAL).

3.4.2. To identify the timestamps for each depolarization event, use a DETECTION SIGNAL, which is a high-pass filtered version of the recorded signal (Butterworth 1st order HPF at 0.01 $s^{-1}$).

3.4.3 By eliminating the low frequency components, the detection of depolarization events can be performed using a simple thresholding method. Use a fixed threshold for noise elimination and event detection (0.3 mV was used for this study).

376    3.4.4. Characterize the depolarization event by a series of thresholds crossings that occur at
377    time intervals smaller than 4 s. Compute the start and the end of depolarization event as
378    determined from consecutive sequences of threshold crossings. Events that are shorter than
379    40 ms can be discarded as noise.
380
381    3.4.5. Compute the amplitude of the events in the unfiltered (RAW SIGNAL) to eliminate
382    errors due to the effect of the low pass filtering on the peak of the event. Select the
383    depolarization wavelet from the raw signal using the timestamps determined in the filtered
384    signal. Measure the amplitude as the difference between the maximum and minimum
385    values of the wavelet selected from the raw signal.
386
387    NOTE: The script files to perform step 3.4 - Depolarization event determination - and to
388    obtain **Figure 1** are provided as **Supplementary File** attached to this article.
389

**REPRESENTATIVE RESULTS**

391    **Figure 1** shows representative voltage traces of 4-6 dpf zebrafish larva extracellular field
392    recordings in the case of two genetic conditions: Mismatch control and *DEPDC5* knock-
393    down. In the baseline period of the recording, *DEPDC5* knock-down shows a higher
394    occurrence of spontaneous events, while the Mismatch control displays very few
395    fluctuations. These activity patterns are representative of the significant increase in
396    neuronal activity due to loss-of-function of *DEPDC5*, as we have previously reported[18]. After
397    PTZ application, both Mismatch control and *DEPDC5* knock-down show an increased
398    number of depolarization events. During the first period after PTZ application (10 – 60 min),
399    a rate of 0.8 events per min is observed in both Mismatch control and *DEPDC5* knock-down,
400    where the majority of events are of high amplitude (> 1 mV). During the latter response
401    period (60 – 120 min after PTZ application), the rate of depolarization events increases to
402    around 1 event per min, and the majority of the events are of low amplitude (≤ 1 mV).
403

**FIGURE LEGENDS:**

405    **Figure 1. Example traces of field recordings in the zebrafish larvae brain. (A)** Overview of
406    180 min recording for a Mismatch control larva and a *DEPDC5* Knock-down. First,
407    spontaneous baseline activity was recorded, then PTZ was applied in bath (red bar). **(B)** Peri-
408    stimulus time histograms of the depolarization events for Mismatch control and *DEPDC5*
409    knock-down. The events were classified as high amplitude (> 1 mV - blue) and low amplitude
410    (≤ 1 mV - black). **(C-E)** Example traces of the different periods of the recording: **(C)**
411    spontaneous activity, **(D)** High amplitude events during the first period after PTZ application,
412    **(E)** Low amplitude events during the latter period after PTZ application. Note that the script
413    files to obtain these figures are provided as **Supplementary File**.
414

415    **Supplementary File. Script files for step 3.4.**
416

**DISCUSSION**

418    Epilepsy is a complex neurological disease, featuring a wide range of etiologies that are
419    starting to be elucidated with the advent of genetic sequencing technologies[25–27]. Versatile
420    animal models are essential for an efficient translational strategy that will yield both insights
421    into the pathological mechanisms of genetically linked epilepsies, as well as targeted
422    therapies for the distinct forms of this condition. Zebrafish models have been very effective

423 at reproducing major features of epilepsy and providing reliable readouts for anti-epileptic
424 drug screening [5,28]. Spontaneous seizures can be detected in genetically modified zebrafish
425 [15,29–31] and neurophysiological analysis in these models[28] has confirmed the neuronal basis
426 of the epileptic-like behavior[32,33]. Small-sized zebrafish larvae are amenable to chemical
427 screens in 96-well format using automated detection of simple behavior, such as
428 spontaneous swimming, which allows for rapid detection of potential therapeutics.
429
430 The *DEPDC5* knock-down model presented here is obtained by injection of AMO into the
431 zebrafish embryo to block gene expression during development. This model presents
432 several keystone phenotypic features during different time points of larval development,
433 which can be used as indicators of therapy efficiency during a chemical or genetic screening
434 protocol. The AMO-mediated gene knock-down is a powerful technique, displaying
435 advantages over chemically-induced seizure models, as it specifically targets the expression
436 of a gene of interest, thus allowing the identification of the underlying pathogenic
437 mechanisms triggered by a genetic mutation. Chemical inducers, which are nevertheless
438 potent tools for drug screenings, can act through multiple cellular pathways that might not
439 be always relevant to the genetic mutation under study. While AMO injection is in itself a
440 simple technique when mastered by the experimenter, it also presents a number of
441 limitations. The injections have to be performed at the one cell stage embryo; in our hands,
442 injections at later stages greatly increased the variability of the phenotype. This limits the
443 time available for injection; therefore, a strategy of generating eggs for injection in a time
444 sequence is useful. We routinely use 4-5 crosses that we open at 15-20 min intervals,
445 allowing the injection of one clutch before obtaining the next one. Further, care must be
446 taken to assess the phenotype at the same time points between different experiments, as
447 stereotyped behaviors evolve rapidly during the first days of development. The volume and
448 concentration of AMOs must also be carefully controlled, as general toxicity due to injecting
449 excessive amounts will mask the specific phenotype. The different controls presented in the
450 introduction are essential for determining the right injection dose and the corresponding
451 phenotype.
452
453 Field recordings of the larval zebrafish brain are a useful tool for investigating the
454 deleterious effects of genetic mutations involved in different brain disorders on the global
455 neuronal activity[34]. Depolarization events seen under these experimental conditions are an
456 established method for assessing electrophysiological effects of drugs in different epileptic
457 conditions[15,35]. However, the assessment of these effects has mostly been done qualitatively
458 rather than quantitatively, and having a subjective observer as an actor in the analysis. Here,
459 we develop an automatic detection strategy that can objectively quantify the rate of
460 depolarizations, their amplitude and duration, and can evaluate the progress of these
461 parameters across time, or with different genetic or pharmacologic interventions.
462
463 The representative results presented here show the expected field activity of the *DEPDC5*
464 knock-down genetic model in comparison to a Mismatch control in 4-6 dpf zebrafish, before
465 and after the application of PTZ to introduce epileptiform-like electrographic activity.
466 Previously, we have shown a significant increase in the basal activity of the *DEPDC5*
467 knockdown condition[18]. Here, we show that the response of these two conditions to PTZ, a
468 chemical epileptiform activity inducer, has a similar trajectory in time, starting with a period
469 of relatively low frequency, high amplitude depolarization events and continuing with a

470 period of higher frequency, lower amplitude depolarization events. Field recording events
471 have slow dynamics (frequencies of interest are in the range of 0.005-0.2 s$^{-1}$), therefore both
472 low-pass and high-pass filters are used in this protocol to isolate the events of interest. After
473 eliminating the low frequency noise, the detection of depolarization events is performed
474 using a simple threshold. Since the statistics of the signal are greatly affected by the
475 presence of depolarization events, we could not use the standard deviation of the total
476 signal to determine this threshold. The variability of the value of the standard deviation
477 across datasets was greater than the observed recording noise levels. Therefore, after visual
478 inspection of the traces, we used a fixed value of the threshold of 0.3 mV, in order to avoid
479 the biasing induced by different levels of depolarization activity.
480
481 The described protocol provides a standardized and simple method for evaluating the motor
482 behavior and the neuronal field activity, via extracellular current clamp voltage recording
483 coupled with automatic detection of depolarization events in the optic tectum, to
484 characterize epileptiform-like phenotypes in zebrafish models.
485

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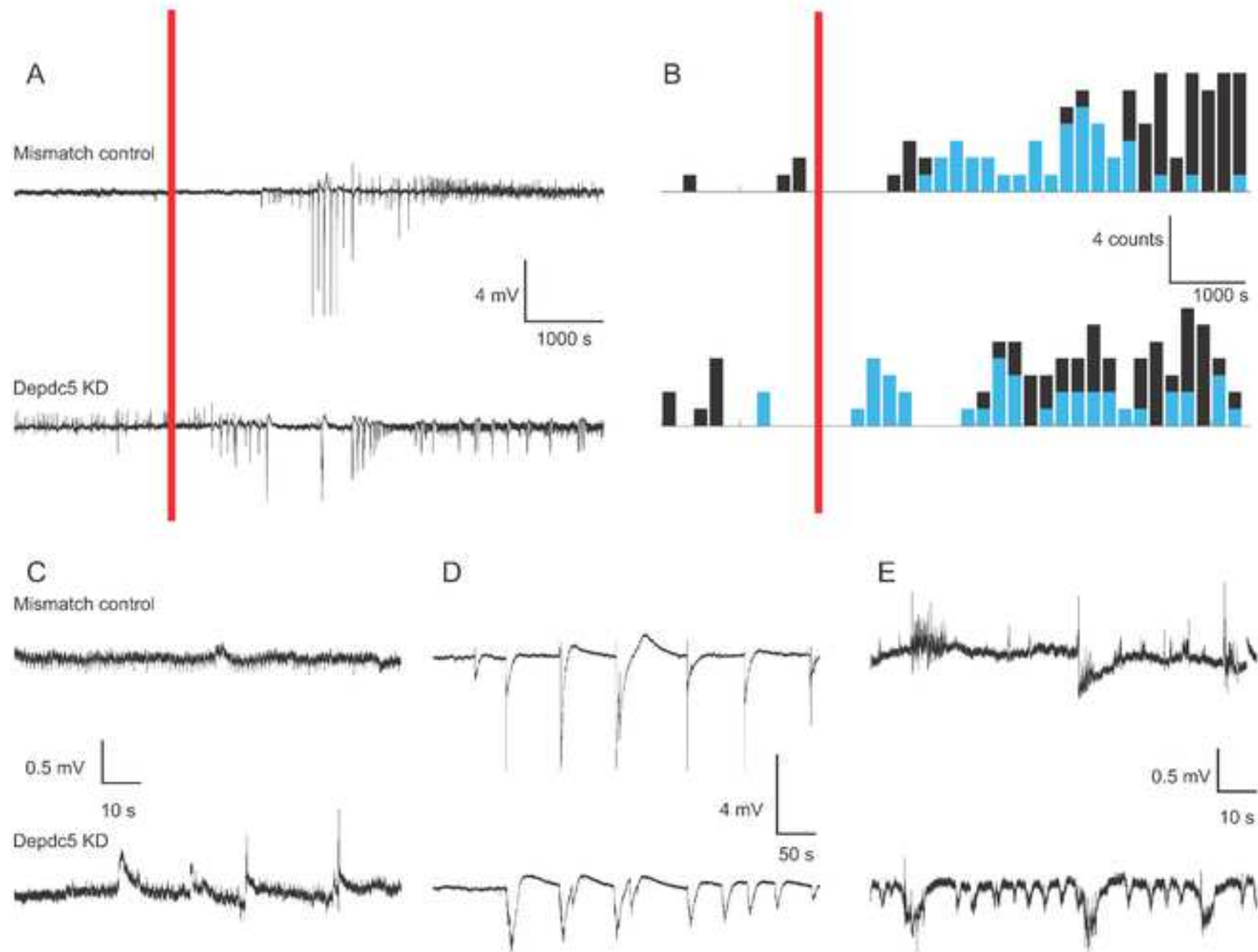
| Name of Material | Company | Catalog Number |
|---|---|---|
| Agarose | Sigma-Aldrich, France | A9539 |
| Aquarium salt | Instant Ocean, Blacksburg, VA | SS15-10 |
| Borosilicate glass with filament | Sutter Instruments | BF100-50-10 |
| CaCl2 | Sigma-Aldrich, France | C1016 |
| Depdc5-atg antisense morpholino | GeneTools, OR, USA | N/A |
| Depdc5-mis antisense morpholino | GeneTools, OR, USA | N/A |
| Depdc5-splice antisense morpholino | GeneTools, OR, USA | N/A |
| Digitizer | Molecular Devices, CA, USA | Digidata 1550 |
| Fast Green Dye | Sigma-Aldrich, France | F7258 |
| Glass-bottom petri dishes | Ibidi, Germany | 81218 |
| glucose | Sigma-Aldrich, France | 68270 |
| Grasshopper 2 camera | FLIR, BC, Canada | GRAS-03K2M-C |
| HEPES | Sigma-Aldrich, France | H3375 |
| human wild-type DEPDC5 cDNA | Dharmacon, France | M_001242897 |
| ImageJ software | NIH, USA | N/A |
| KCl | Sigma-Aldrich, France | P9333 |
| Matlab software | MathWorks, MA, USA | N/A |
| MgCl2 | Sigma-Aldrich, France | M2670 |
| NaCl | Sigma-Aldrich, France | S7653 |
| NaOH | Sigma-Aldrich, France | 71687 |
| Pancuronium bromide | Alomone Labs | P-130 |
| Parafilm | Sigma-Aldrich, France | P7793 |
| Patch clamp amplifier | Molecular Devices, CA, USA | MultiClamp 700 |
| pClamp10 acquisition software | Molecular Devices | N/A |
| Pentylenetetrazol (PTZ) | Sigma-Aldrich, France | P6500 |
| Pipette puller | Narishige, Japan | PC-10 |
| Pneumatic PicoPump | WPI, France | PV 820 |
| Sylgard 184 kit | Sigma-Aldrich Intl. | 761036 |
| Transfer plastic pipettes | Sigma-Aldrich, France | Z350605 |
| Zebralab | Viewpoint, France | N/A |
| | | |

| Comments/Description |
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| OD: 1,5mm, ID: 0,5 mm |
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| sequence 5'- TGCCTTCATGGTGACCGTCATTTTA -3' |
| sequence 5'- TGCgTTgATcGTGACCcTgATTTTA -3' |
| sequence 5'- ACATTCCTGTTTCACCATAGATGAT -3' |
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| Stock solution of 0.2% |
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| formerly Point Grey Research |
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| Stock solution of 60mM in water |
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| Computer-controled patch clamp amplifier |
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| Stock solution of 300mM (dissolved in recording solution) |
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Department: *Neuroscience*

Institution: *Université Sorbonne*

Article Title: *Behavioral and physiological characterization of a zebrafish epilepsy model*

Signature: *[signature]*

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*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 ».

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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).

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**Supplemental Coding Files**

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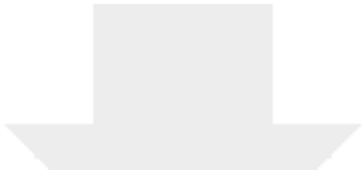
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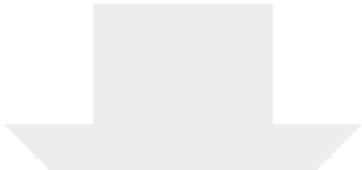**Supplemental Coding Files**
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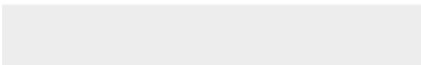
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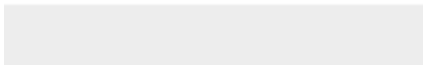
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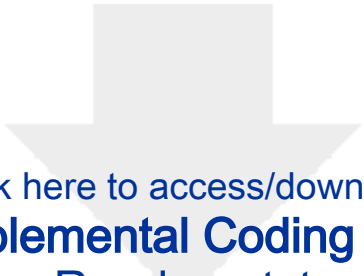
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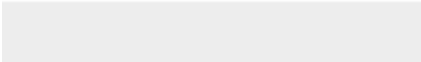
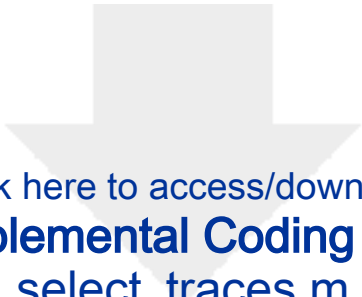**Supplemental Coding Files**

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