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TITLE:

A Scalable Model to Study the Effects of Blunt-Force Injury in Adult Zebrafish

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KEYWORDS:

zebrafish, regeneration, traumatic brain injury, blunt-force trauma, zebrafish brain, seizure, edema, proliferation

SUMMARY:

We modified the Marmarou weight drop model for adult zebrafish to examine a breadth of pathologies following blunt-force traumatic brain injury (TBI) and the mechanisms underlying subsequent neuronal regeneration. This blunt-force TBI model is scalable, induces a mild, moderate, or severe TBI, and recapitulates injury heterogeneity observed in human TBI.

ABSTRACT:

Blunt-force traumatic brain injuries (TBI) are the most common form of head trauma, which spans a range of severities and results in complex and heterogenous secondary effects. While there is no mechanism to replace or regenerate the lost neurons following a TBI in humans, zebrafish possess the ability to regenerate neurons throughout their body, including the brain. To examine the breadth of pathologies exhibited in zebrafish following a blunt-force TBI and to study the mechanisms underlying the subsequent neuronal regenerative response, we modified the commonly used rodent Marmarou weight drop for the use in adult zebrafish. Our simple blunt-force TBI model is scalable, inducing a mild, moderate, or severe TBI, and recapitulates many of the phenotypes observed following human TBI, such as contact- and post-traumatic seizures, edema, subdural and intracerebral hematomas, and cognitive impairments, each displayed in an injury severity-dependent manner. TBI sequelae, which begin to appear within minutes of the injury, subside and return to near undamaged control levels within 7 days post-injury. The regenerative process begins as early as 48 hours post-injury (hpi), with the peak cell proliferation observed by 60 hpi. Thus, our zebrafish blunt-force TBI model produces characteristic primary

and secondary injury TBI pathologies similar to human TBI, which allows for investigating disease onset and progression, along with the mechanisms of neuronal regeneration that is unique to zebrafish.

INTRODUCTION:

Traumatic brain injuries (TBIs) are a global health crisis and a leading cause of death and disability. In the United States, approximately 2.9 million people experience a TBI each year, and between 2006–2014 mortality due to TBI or TBI sequelae increased by over 50%[1]. However, TBIs vary in their etiology, pathology, and clinical presentation due largely in part to the mechanism of injury (MOI), which also influences treatment strategies and predicted prognosis[2]. Though TBIs can result from various MOI, they are predominately the result of either a penetrating or blunt-force trauma. Penetrating traumas represent a small percentage of TBIs and generate a severe and focal injury that is localized to the immediate and surrounding impaled brain regions[3]. In contrast, blunt-force TBIs are more common in the general population, span a range of severities (mild, moderate, and severe), and produce a diffuse, heterogeneous, and global injury affecting multiple brain regions[1,4,5].

Zebrafish *(Danio rerio)* have been utilized to examine a wide range of neurological insults spanning the central nervous system (CNS)[6–9]. Zebrafish also possess, unlike mammals, an innate and robust regenerative response to repair CNS damage[10]. Current zebrafish trauma models use various injury methods, including penetration, excision, chemical insult, or pressure waves[11–16]. However, each of these methods utilizes an MOI that is rarely experienced by the human population, is not scalable across a range of injury severities, and does not address the heterogeneity or severity-dependent TBI sequela reported after blunt-force TBI. These factors limit the use of the zebrafish model to understand the underlying mechanisms of the pathologies associated with the most common form of TBI in the human population (mild blunt-force injuries).

We aimed to develop a rapid and scalable blunt-force TBI zebrafish model that provides avenues to investigate injury pathology, progression of TBI sequela, and the innate regenerative response. We modified the commonly used rodent Marmarou[17] weight drop and applied it to adult zebrafish. This model yields a reproducible range of severities ranging from mild, moderate, to severe. This model also recapitulates multiple facets of human TBI pathology, in a severity-dependent manner, including seizures, edema, subdural and intracerebral hematomas, neuronal cell death, and cognitive deficits, such as learning and memory impairment. Days following injury, pathologies and deficits dissipate, returning to levels resembling undamaged controls. Additionally, this zebrafish model displays a robust proliferation and neuronal regeneration response across the neuroaxis concerning injury severity.

Here, we provide details toward the set up and induction of blunt-force trauma, scoring post-traumatic seizures, assessment of vascular injuries, instructions on preparing brain sections, approaches to quantifying edema, and insight into the proliferative response following injury.

PROTOCOL:

Zebrafish were raised and maintained in the Notre Dame Zebrafish facility in the Freimann Life Sciences Center. The methods described in this manuscript were approved by the University of Notre Dame Animal Care and Use Committee.

1. Traumatic brain injury paradigm

1.1 Add 1 mL of 2-phenoxyethanol to 1 L of system water (60 mg of Instant Ocean in 1 L of deionized RO water).

1.2 Prepare an aerated recovery tank containing 2 L of system water at room temperature.

1.3 Select the desired weight of ball bearing and a desired length and diameter of steel/plastic tubing and determine the energy and impact force.

NOTE: Tubing should have an inner diameter that allows the ball bearing to pass through without changing its path or speed of movement.

1.3.1. Determine the kinetic energy upon impact:

$$KE = mgh$$

Where, KE = kinetic energy, m = mass (in kg), g = gravitational force, h = height (in m) from drop point to fish.

NOTE: This provides kinetic energy in J. Multiply the value by 1,000 to determine mJ. KE is based on an accelerating object, which occurs when the ball bearing is dropped from a stationary position.

1.3.2 Generate a mild TBI (miTBI) using 7.62 cm long steel/plastic tubing that ends 1.5 cm above the plate on the zebrafish skull (total distance 9.1 cm) and a 1.5 g (6.4 mm diameter) ball bearing. These produce a kinetic energy of 1.33 mJ. This damage was empirically decided to be equivalent to a miTBI based on key pathophysiological TBI markers, such as vascular injury, subdural/intracerebral hematoma formation, neuronal cell death, and cognitive impairments that largely recapitulated what was reported in the human population in a severity-dependent manner.

1.3.3 Calculate kinetic energy miTBI = $0.0015kg * \frac{9.8m}{s^2} * 0.091m = 0.00133J * 1000 = 1.33mJ$

1.3.4 Generate a moderate TBI (moTBI) using 12.7 cm long steel/plastic tubing that ends 1.5 cm above the plate on the zebrafish skull (total distance 14.2 cm) and a 1.5 g (6.4 mm diameter) ball bearing that produces kinetic energy of 2.08 mJ.

1.3.5 Calculate kinetic energy moTBI = $0.0015kg * \frac{9.8m}{s^2} * 0.142m = 0.00208J * 1000 = 2.08mJ$

1.3.6 Generate a severe TBI (sTBI) using 7.62 cm long steel/plastic tubing that ends 1.5 cm above the plate on the zebrafish skull (total distance 9.1 cm) and a 3.3 g (8.38 mm diameter) ball bearing that produces a kinetic energy of 2.94 mJ.

1.3.7 Calculate kinetic energy sTBI = $0.0033kg * \frac{9.8m}{s^2} * 0.091m = 0.00294J * 1000 = 2.94mJ$

1.4 Fill a Petri dish with modeling clay (**Figure 1**, step 1) and use a blunt instrument (i.e., the back of a pair of forceps) to create a raised platform (5 cm x 1.5 cm) with additional modeling clay (**Figure 1**, step 2).

1.4.1 Use a razor blade to divide the raised platform lengthwise into two approximately equal halves (**Figure 1**, step 3, red dashed line). Form the two halves into a channel that accommodates the length of an adult fish (**Figure 1**, step 4). Use additional clay to build walls that will secure ~2/3 of the fish body, leaving the head exposed.

1.4.2 Mold a small support in the exposed head region perpendicular to the walls to support the head to avoid rotation or recoil of the head upon injury (**Figure 1**, step 4).

NOTE: The channel should be deep enough to support the fish in a dorsal-up position but should still allow the head to rest above the surrounding clay. Additionally, the head support should follow the natural curvature of the fish, supporting the lower mandible and gills.

1.4.3 Ensure that the dropped weight is not impeded by the channel's sides (**Figure 1**, step 4).

1.5 Create a 3 mm diameter steel disk using a mini hole-punch and 22 g steel flashing. Each disk may be used multiple times.

1.6 Anesthetize one fish by placing it into a beaker with 50–100 mL of 1:1000 (1 mL/1 L, 0.1%) 2-phenoxyethanol until it is unresponsive to tail pinch.

1.7 Place the fish, dorsal side up, on the clay mold within the channel so that the body is secured on the sides and place a 3 mm 22 g steel disc on the head, centered over the desired impact point (**Figure 1**, step 5).

1.7.1. Ensure that the fish is aligned as perpendicularly as possible to avoid its head from tilting to one side, which could cause an uneven impact.

1.8 Secure the steel/plastic tubing, using a standard ring stand and arm clamp, so the bottom of the tubing is 1.5 cm above the head of the zebrafish (**Figure 1**, step 6). Ensure the tubing is straight.

1.8.1 Look down the tubing and ensure that the tubing is aligned above the steel plate.

1.9 Drop the ball bearing (1.5 g for mild and moderate TBI, and 3.3 g for severe TBI) from a predetermined height (described in Steps 1.3.3-1.3.5) down the tubing onto the steel plate located over the desired neuroanatomical region of interest (e.g., cerebellum, **Figure 1**, step 5) to produce the blunt-force TBI for the desired severity of injury. Place the injured fish in a recovery tank to be monitored.

NOTE: Depending on the severity of the injury, mortality or tonic-clonic seizures may occur. If the fish are unresponsive for a prolonged period of time following the TBI, use a transfer pipette or forceps to administer a tail-pinch and evaluate for a pain response.

2. Scoring seizures post-TBI in the adult zebrafish

2.1 Anesthetize and injure the fish according to the injury protocol outlined in section 1 and place the injured fish in an aerated recovery tank of 2 L of system water.

2.2 Observe the fish for any signs of post-traumatic seizures beginning immediately after being placed in the recovery tank. Set an observation time (i.e., 1 h) and record all seizure activity, including the seizure score (described below), duration of each seizure, and the percentage of fish that experienced seizures (**Figure 2A**).

NOTE: Seizures can occur immediately upon injury, as well as hours or days post-TBI. Seizures can persist long term and a single fish can have multiple seizures. Setting an observation time of 1 h or greater will yield a good representation of the overall trend of seizure rates.

2.3 Score fish using the Mussulini[18] guidelines for adult zebrafish seizure phenotypes.

NOTE: Without using the tracking software, it is difficult to assess seizure scores below 3 unbiasedly. Therefore, only seizure scores of 3 or greater should be recorded when the assessment is being performed without software.

3. Brain dissection

3.1 Euthanize fish in a 1:500 (2 mL/1 L, 0.2%) solution of 2-phenoxyethanol, until gill movements cease, and they are unreactive to fin pinch, at the desired endpoint.

3.2 Fill a Petri dish with modeling clay and create a small cavity to support the body during dissection.

3.3 Place the fish, dorsal side up, in the clay mold. Place one dissection pin through the midline halfway down the body of the fish and a second pin ~5 mm behind the base of the head.

3.4 Under a dissecting light microscope, bluntly sever the optic nerve with a pair of #5 Dumont forceps and remove the eyes (**Figure 2A**).

3.5 Orientate the fish so that the rostral end is furthest when looking through the microscope (**Figure 2B**).

NOTE: The following steps are for right-handed individuals. Left-handed individuals may prefer to perform the following steps in a mirrored orientation.

3.6 Use #5 forceps to slowly place one end of the forceps under the right parietal plate, making a deliberate scissor action moving toward the rostral end and remove the right parietal and frontal plates (**Figure 2C**).

3.6.1. Keep the forceps at an angle of 45˚ or lower in order to avoid penetrating the brain during dissection.

3.7 Rotate the fish 90˚ clockwise. Place one end of the #5 forceps under the left parietal plate and use the same scissor motion to remove the left parietal and frontal plates exposing the entire dorsal aspect of the brain (**Figure 2D,E**).

3.8 Bluntly transect the maxilla with #5 forceps. Preserve the olfactory bulbs and do not damage them if this is the region of interest.

3.9 Remove the right opercle, preopercle, interopercle, and subopercle with #5 forceps (**Figure 2F**).

3.10 Bluntly resect the musculature at the caudal end of the calvarium opening using #5 forceps, exposing the spinal cord.

3.11 Bluntly transect the spinal cord with #5 forceps. Carefully place forceps under the brain and gently remove the brain from the calvarium.

NOTE: Never pinch the brain. Use forceps to “cradle” the brain or resect caudally and utilize the exposed spinal cord as a pinch point to maneuver the brain.

3.12 Fix the removed brains in 9 parts 100% ethanol to 1 part 37% formaldehyde overnight at 4˚C on a rocker platform.

4. Edema studies in the zebrafish brain

4.1 Anesthetize and injure fish according to the injury protocol outlined in section 1 and allow the fish to recover in a recovery tank until they start swimming freely.

4.2 Place fish back in the normal housing conditions post-injury for 1 day.

4.3 Euthanize the fish in 1:500 2-phenoxyethanol, after time has elapsed.

4.4 Dissect the whole brain or region of interest according to the protocol outlined in section 3 and place the brain immediately on a small weigh boat.

NOTE: Use caution when transferring the brain, using fine forceps to gently place it on the weigh boat without stabbing or scraping the brain, which could result in the loss of tissue.

4.5 Label (with injury group and brain number) and tare an additional small drying weigh boat on a scale. Use a scale with the ability to measure a minimum of 0.001 g to get an accurate measurement.

4.6 Transfer the brain to the tared drying weigh boat and record the wet weight of the brain. Orient the brains so that they lay flat on the weigh boat with the dorsal side facing up.

4.7 Place the drying weigh boat and brain in a hybridization oven set to 60 ˚C for 8 h.

4.8 After drying, the brain may stick to the drying weigh boat and may be difficult to remove and transfer to a new tared small weigh boat. Avoid pinching the brain with forceps, as this might result in damage to the dry brain and loss of tissue. Instead, pinch the fine forceps together and, starting at the ventral side of the brain, scoop in an upward motion.

4.9 Determine the water content of each brain using the formula (**Figure 4**):

$$\%\, Water\ Content = \frac{wet\ weight - dry\ weight}{wet\ weight} * 100$$

5. Labeling cellular proliferation across the neuroaxis and preparing fixed tissue.

5.1 Prepare 10 mM 5-ethynyl-2'-deoxyuridine (EdU) in 2 mL of ddH_2O.

5.2 Anesthetize 3 to 4 fish at a time in 50–100 mL of 1:1000 (1 mL/1 L, 0.1%) 2-phenoxyethanol until the fish are unresponsive to tail pinch, at the desired time point following injury (using the protocol outlined in section 1).

5.3 Make a partial incision on a wet sponge and place one fish at a time in the opening, ventral side up.

5.4 Use a 30 G needle to inject ~40 µL of 10 mM EdU into the fish's body. Return the fish to a holding tank filled with system water.

NOTE: Repeated injections may be performed at different time points to label a greater number of proliferating cells and may be needed if a chase period of greater than 1 week is desired.

5.5 Collect brains as outlined in section 3 and place them as a group in a 5 mL glass vial containing 2 mL of 9 parts 100% ethanol to 1 part 37% formaldehyde. Fix the brains at 4 ˚C on a rocker platform.

NOTE: A rocker or shaker platform prohibits the brains from resting at the bottom and the telencephalon of whole brains from curling.

5.6 Rehydrate brains, in the same glass vial used to fix brains, in washes of descending ethanol series, 75%, 50%, and 25%, for 15 mins each, followed by a 1.5 h wash in 5% sucrose/PBS on a rocker at room temperature. Store the brains in a glass vial overnight in 30% sucrose/PBS at 4˚ C on a rocker platform.

5.7 Remove brains from 30% sucrose/PBS and transfer them with forceps into a 12-well plate (one treatment group per well) with wells filled with a 2:1 solution consisting of 2 parts tissue freezing medium and 1 part 30% sucrose/PBS. Incubate the brains overnight at 4 ˚C on a rocker platform.

5.8 Transfer brains to the next row of wells within the 12-well plate submerging brains in 100% TFM for 2–24 h at 4 ˚C.

5.9 Use a cryostat chuck to embed the brains in TFM in the desired orientation on dry ice.

5.10 Perform cryosectioning (16 μm thick sections) and collect the sections on positively charged slides. Dry slides on a slide warmer for 1 h and then store at -80 ˚C or continue with immunohistochemistry.

5.11 Prepare a hydrophobic barrier on the slide around the tissue sections and allow to dry on a slide warmer for 20 min.

5.12 Briefly wash the slides in PBS for 5 min and then twice in PBS-Tween 20 (0.05%) for 10 min each.

5.13 Perform EdU detection using the EdU Cell Proliferation Kit and the manufacturer's instructions.

5.14 Analyze the slides and quantify the fluorescent EdU-labeled cells using either an epifluorescent microscope or a confocal microscope. A minimum of a 40x objective will be required to clearly distinguish individual cells.

REPRESENTATIVE RESULTS:

Preparing the injury-induction rig allows for a rapid and simplistic means of delivering a scalable blunt-force TBI to adult zebrafish. The graded severity of the injury model provides several easily identifiable metrics of successful injury, though the vascular injury is one of the easiest and most prominent pathologies (**Figure 3**). The strain of fish used during the injury can make this indicator

easier or harder to identify. When using *wild-type AB* fish (*WT*^{AB}, **Figure 3A–D**), identification of vascular injury can be difficult to distinguish between either miTBI or moTBI and undamaged control fish due to the pigmentation (**Figure 3A–C**). Following injury, miTBI fish display minimal surface abrasions (**Figure 3B**), while moTBI exhibit limited cerebral hemorrhaging (**Figure 3C**). While sTBI can still be challenging, the extent of injury is often apparent (**Figure 3D**). In contrast, when using *albino* (**Figure 3E–H**) or *casper* fish (**Figure 3I–L**), vascular injury is easily identified. Additionally, impact seizures are often observed following injury and seizure rate among the group is another representative metric of injury (**Table 1**). Injured fish will display tonic-clonic seizures (ataxia, ZBC 1.9, bending, ZBC 1.16, circling, ZBC 1.32, and corkscrew swimming, ZBC 1.37)[19] that are easily observed following injury, regardless of the background strain. Seizures will be observed with increasing prevalence in relation to severity. After injury, miTBI do not display seizure-like behaviors; however, moTBI will display seizure behaviors (10.66% ± 1.37%, $p < 0.0001$, **Table 1**) and the incidence is further elevated in sTBI fish (19.93% ± 1.49%, $p < 0.0001$, **Table 1**).

Successful removal of the brain is critical for a myriad of further investigations, such as edema and assessing cell proliferation. Perform dissections with the utmost care to avoid damaging brain regions (most often by unintentional puncture) and to conserve all regions (the olfactory bulbs can easily be lost). Following the brain dissection procedure and schematic (section 3, **Figure 2A–F**) allows for a complete brain removal (**Figure 2G,H**). Investigators should consider whether their analysis requires the entire brain or whether a collection of specific brain regions may suit their needs. Dependent on the severity of the injury and time of collection, the brains may exhibit attached subdural hemorrhages, however, these are often adhered to the underside of the skull and lost during dissection. Swelling of the cerebrum is at times apparent, but due to anatomical differences and variation in general size, edema is the best method to assess swelling. Following the protocol outlined (section 4), undamaged brains exhibit a fluid content of 73.11% ± 0.80%, and miTBI, though slightly elevated, do not display a significant increase in edema at 1, 3, or 5 dpi (1 dpi: 76.33% ± 1.32%, $p = 0.36$, 3 dpi: 75.33 ± 1.37%, $p = 0.84$, 5 dpi: 74.14 ± 1.50%, $p > 0.99$, **Figure 4**). In contrast, both moTBI and sTBI had significant edema 1 dpi (moTBI: 80.55 ± 0.94%, $p < 0.0001$, sTBI: 86% ± 1.05%, $p < 0.0001$), and 3 dpi (moTBI: 78.11 ± 0.93%, $p < 0.018$, sTBI: 77.77% ± 1.02%, $p < 0.036$, **Figure 4**). However, fluid content of both moTBI and sTBI returned to levels resembling undamaged controls by 5 dpi (moTBI: 74.42 ± 1.25%, $p > 0.99$, sTBI: 73.85% ± 1.01%, $p > 0.99$, **Figure 4**).

Cell proliferation, following TBI in zebrafish, is a robust assessment of the extent of injury. While the cell proliferation response has been studied previously in zebrafish following other forms of brain injury[9,12], in most instances, the investigation was limited to the injury site. This blunt-force TBI results in a robust proliferation response spanning the neuroaxis. In a severity-dependent manner (sTBI data shown), increased EdU labeling is observed in the ventricular and subventricular zones of the forebrain (telencephalon, **Figure 5B**) relative to undamaged controls (**Figure 5A**). As sections moved caudally into the midbrain (mesencephalon and diencephalon), injured brains displayed increased EdU labeling in the periventricular grey zone (PGZ) the optic tectal lobes (TeO), and aspects of the anterior hypothalamus compared to undamaged fish (**Figure 5D** and **Figure 5C**, respectively). In the hindbrain, neurogenic regions that are evident in

the undamaged brain (**Figure 5E,G**) exhibit increased cell proliferation following the sTBI (**Figure 5F,H**).

To summarize, a modified Marmarou weight drop applied to adult zebrafish provides a reproducible and scalable mild, moderate, or severe blunt-force TBI. Zebrafish, in a severity-dependent manner, display various pathologies, including seizures and vascular injury (i.e., subdural and intracerebral hematomas). Additionally, injured fish display decreased recovery rate (analogous to loss of consciousness, cognitive deficits in the form of learning and memory issues, and neuronal cell death (data not shown). The pathologies observed, rapidly recover over the span of 4–7 days coinciding with robust proliferative events across the neuroaxis.

FIGURE AND TABLE LEGENDS:

Figure 1: Setting up the scalable injury apparatus. Graphical representation of the setup, the model, and delivery of scalable TBIs to the zebrafish. Steps 1–4 provide instructional overview of the steps to form the support mold that immobilizes the fish and exposing the head during damage. Steps 5–7 provide instructions on delivering the injury with insight on aspects to consider when troubleshooting the model. The figure was created with BioRender.com.

Figure 2: Skull removal for brain dissection. Schematic of a simplified zebrafish skull and the step-by-step removal of bone (blue sections) to expose the adult zebrafish brain. (**A,B**) The eyes are bluntly removed with #5 forceps severing the optic nerves. (**C**) Forceps are placed into the musculature directly caudal to the parietal plates (black arrow) to remove the right parietal bone and then the right frontal bone. (**D**) The left parietal bone and left frontal bone are removed. (**E**) The right opercle, preopercle, interopercle, and subopercle are removed, providing lateral and dorsal access to the brain. (**G,H**) Undamaged and sTBI brains were removed. Scale bar = 500 µm.

Figure 3: Vascular injury in various backgrounds across injury-severities. Dorsal view of undamaged and TBI *wild-type AB, albino^{b4}, and casper* adult zebrafish displaying vascular injury. (**A–D**) Adult *wild-type AB* fish are heavily pigmented and abrasions following miTBI (**B**) are difficult to visualize. Vascular injury was more apparent in moTBI (**C**) and sTBI fish compared to undamaged controls (**A**). (**E–H**) *albino* fish were less pigmented and visualization of the brain was more distinct. Vascular injury following TBI was clearly observed and distinguished across severities. (**I–L**) *casper* fish provided the most adaptable background for novice investigators as the transparency allowed for easy identification of desired neuroanatomical regions and clear observation and delineation of vascular injury by TBI-severity. Scale bar = 500 µm.

Figure 4: Zebrafish experience injury-induced edema following TBI. Zebrafish were exposed to different severities of TBI (undamaged, miTBI, moTBI, and sTBI) and assessed at different days following damage for percent fluid content (edema). Statistical analyses were performed with a Browns-Forsythe and Welch ANOVA followed by a Dunnett's T3 multiple comparison post-hoc test. n = total number of individual fish. All statistical analyses were performed with the Prism (Graphpad 9.0) software package.

Figure 5: TBI-induced proliferation across the neuroaxis. (**A–H**) Confocal images of coronal and sagittal brain sections of undamaged and sTBI fish that were IP-injected with EdU 12 h prior to collection. Increased EdU incorporation was observed in multiple neurogenic niches following injury across the forebrain (**A,B**), midbrain (**C,D**), and hindbrain (**E–H**). Cerebellum, CCe, Granule Layer, GL, Medial Valvula Cerebelli, Vam, Molecular Layer, ML, Optic Tectum, TeO, Periventricular Grey Zone, PGZ, Telencephalon, and Tel. All scale bars are 200 μm.

Table 1. Zebrafish display severity-dependent impact seizures following TBI. Quantification of tonic-clonic seizures, which was recorded as the percent of an experimental injured group, that were observed within 1 h post-injury. Statistical analyses were performed with a one-way ANOVA followed by Tukey's post-hoc test. N = total number of experimental groups, n = total number of individual fish. Statistical analyses were performed using the Prism (Graphpad 9.0) software package.

DISCUSSION:

Investigations of neurotrauma and associated sequelae have long been centered on traditional non-regenerative rodent models[20]. Only recently have studies applied various forms of CNS damage to regenerative models[9,11,13,14,21]. Though insightful, these models are limited by either their use of an injury method uncommonly seen in the human population (penetrating traumas, chemical ablation, blast) and/or the injury is not scalable and therefore does not fully address the heterogeneity of severity-dependent pathologies observed in the human population[22–25]. Here, we provide a damage paradigm that applies the most common form of clinically relevant head trauma (blunt-force)[4] that produces many of the pathological metrics established in human diagnosis[22–25]. When applied to the regenerative zebrafish, the model provides avenues to investigate the progression and recovery of injury-induced pathologies across severities, such as edema or post-traumatic seizures, as well as elucidating the mechanisms behind the innate regenerative recovery.

There are two key features of our model in producing a blunt-force TBI in zebrafish. First, our model delivers an inexpensive and simple injury paradigm that is rapid, which permits the successive injury to a large number of individuals or repeated injury to an individual to investigate the cumulative effect of blunt-force TBI. Second, this model is easily scalable to examine the effects of different force impacts. By changing the length of the tube (the height from which the ball bearing is dropped) and the weight of the ball bearing, the energy delivered to the skull of the fish, and the impact force can be easily modified and computed. This scalability of the injury allows for multiple avenues of investigation in regard to severity-dependent TBI sequelae progression and regenerative mechanisms of CNS repair.

With multiple metrics to access successful injury application, careful consideration should still be given to the genetic background of fish to be utilized. The *casper* or *albino* mutant fish will be favorable for novice investigators to reliably place the fish under the drop shaft, the placement of the steel disk over the desired neuroanatomical impact point, and assessing vascular injury. Furthermore, careful removal of the brain is simplified by the visual accessibility of the bones and brain in the *casper* and *albino* mutant fish. However, pigmented wild-type fish can be used

though identification of landmarks and successful dissection may come with notable practice. Furthermore, pigmented fish can be used when producing either a moTBI or sTBI insult, as the subsequent pathologies allow for proper characterization of injury.

One major reason to study the effects of blunt-force TBI in zebrafish is to examine the source of injury-induced cell proliferation and the mechanisms underlying neuronal regeneration. Developmental and basal levels of constitutive proliferation have been identified in neurogenic niches across the zebrafish neuroaxis[26,27], and injury-induced regeneration has been observed localized or adjacent to the injury site in adult zebrafish[8,12,15]. However, our blunt-force TBI model demonstrates that the diffuse injury also results in a severity-dependent cell proliferation event within the neurogenic niches across the neuroaxis. Identification of the source and extent of cell proliferation following TBI will permit the application of single-cell RNA-Seq to identify the changes in gene expression in the proliferative niche and testing the role of different signaling pathways, through the application of specific agonists and antagonists, in regulating this regeneration response. This approach has proven useful in elucidating the mechanisms underlying neuronal regeneration in the damaged zebrafish retina[28] and should be equally useful in the brain following TBI.

In conclusion, our model provides a rapid, simple, and cost-effective injury method to deliver a scalable blunt-force TBI. This model will be useful to further investigate the effects of severity-dependent or repeated blunt-force TBI, as well as elucidating therapeutic targets of genetic regulation improving neuronal protection or inducing neuronal regeneration for functional cognitive recovery in adult vertebrates.

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DISCLOSURES:
The authors have nothing to disclose.

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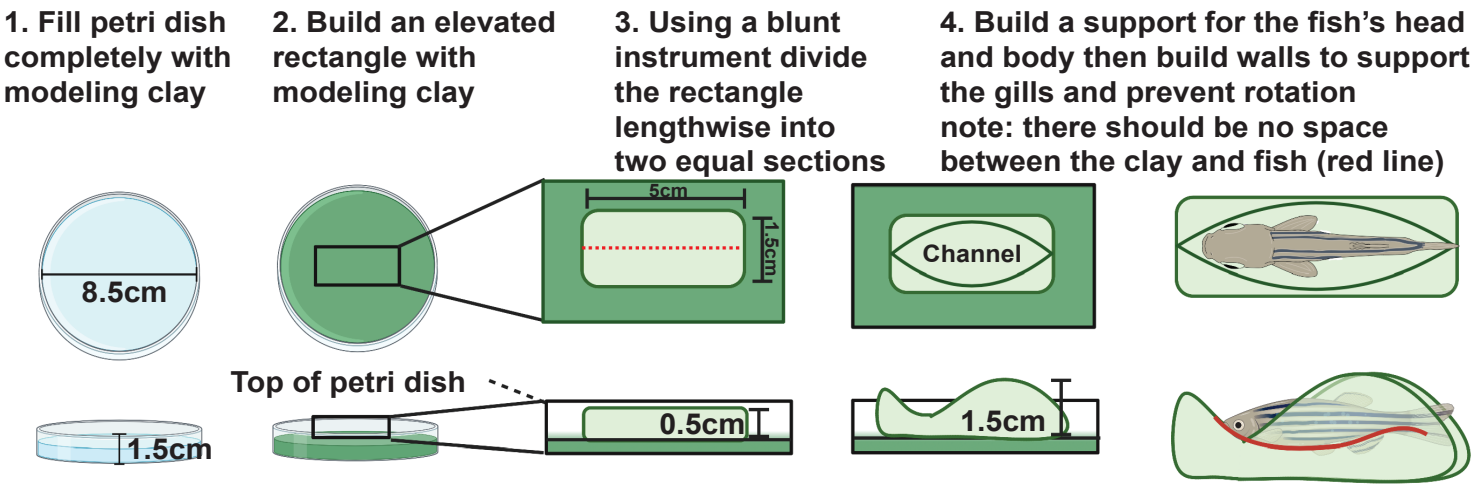
Figure 1

Click here to access/download;Figure;Figure 1. Basic Procedure.pdf



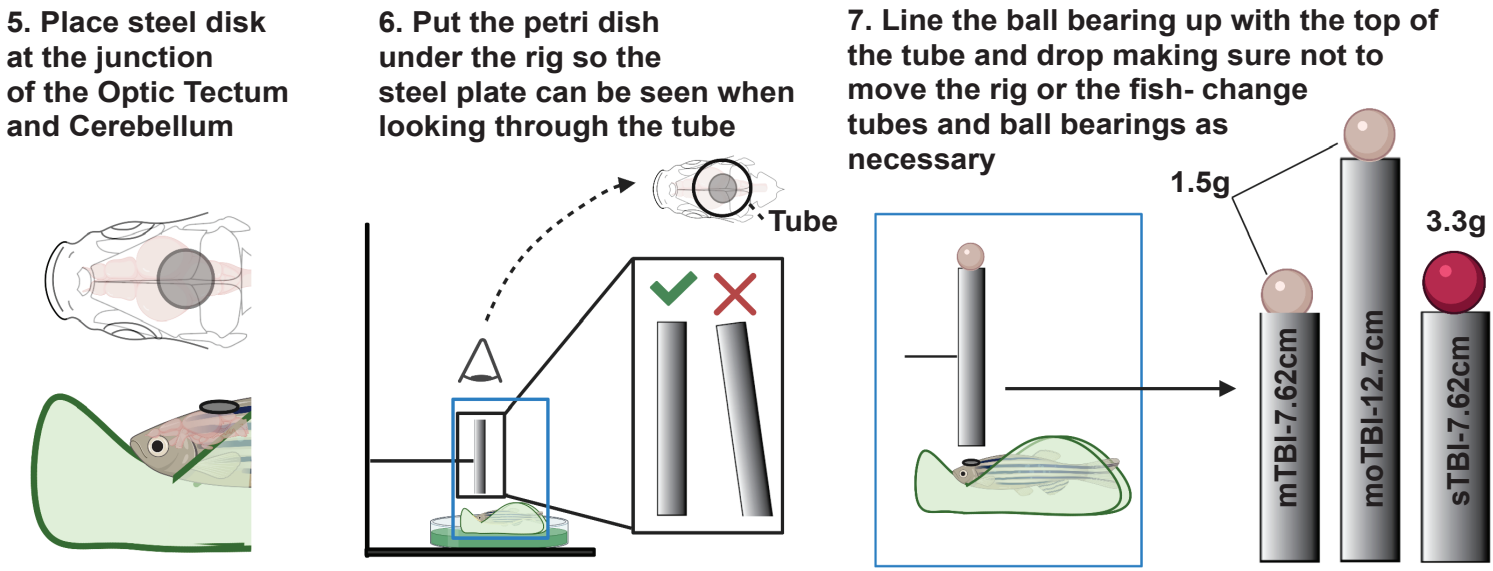


Figure 2

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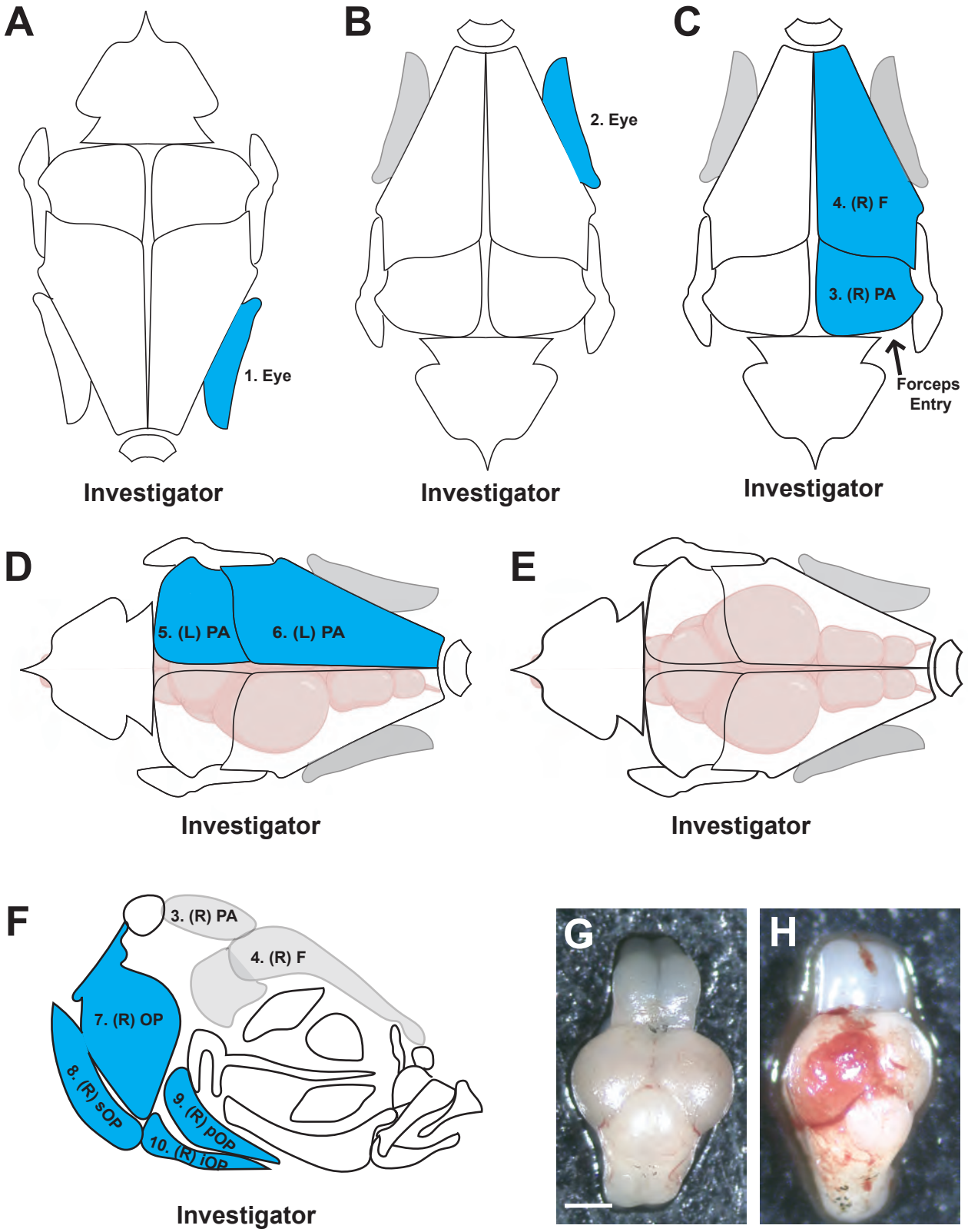

Figure 3

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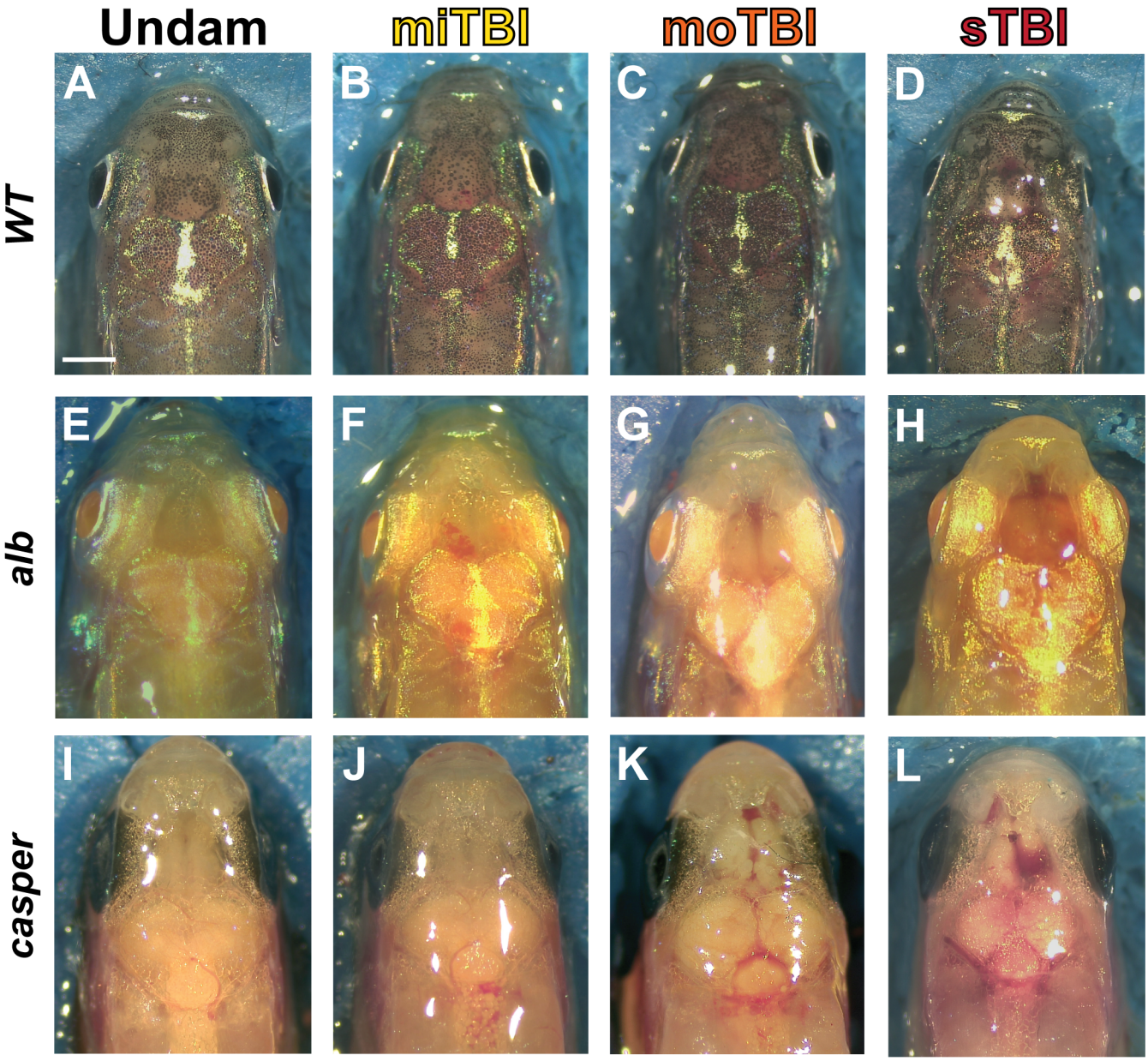

Figure 4

Click here to access/download;Figure;joVE Figure 4 Edema.pdf

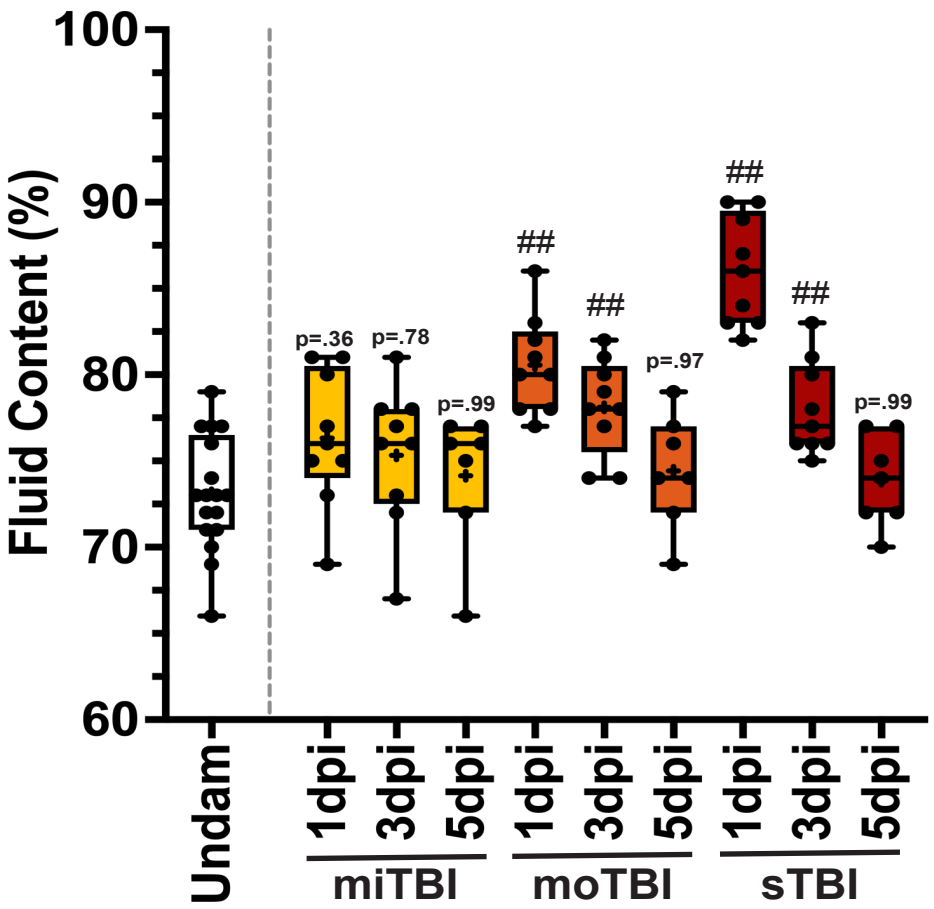

Figure 5

Click here to access/download;Figure;Figure 5 TBI proliferation2.pdf

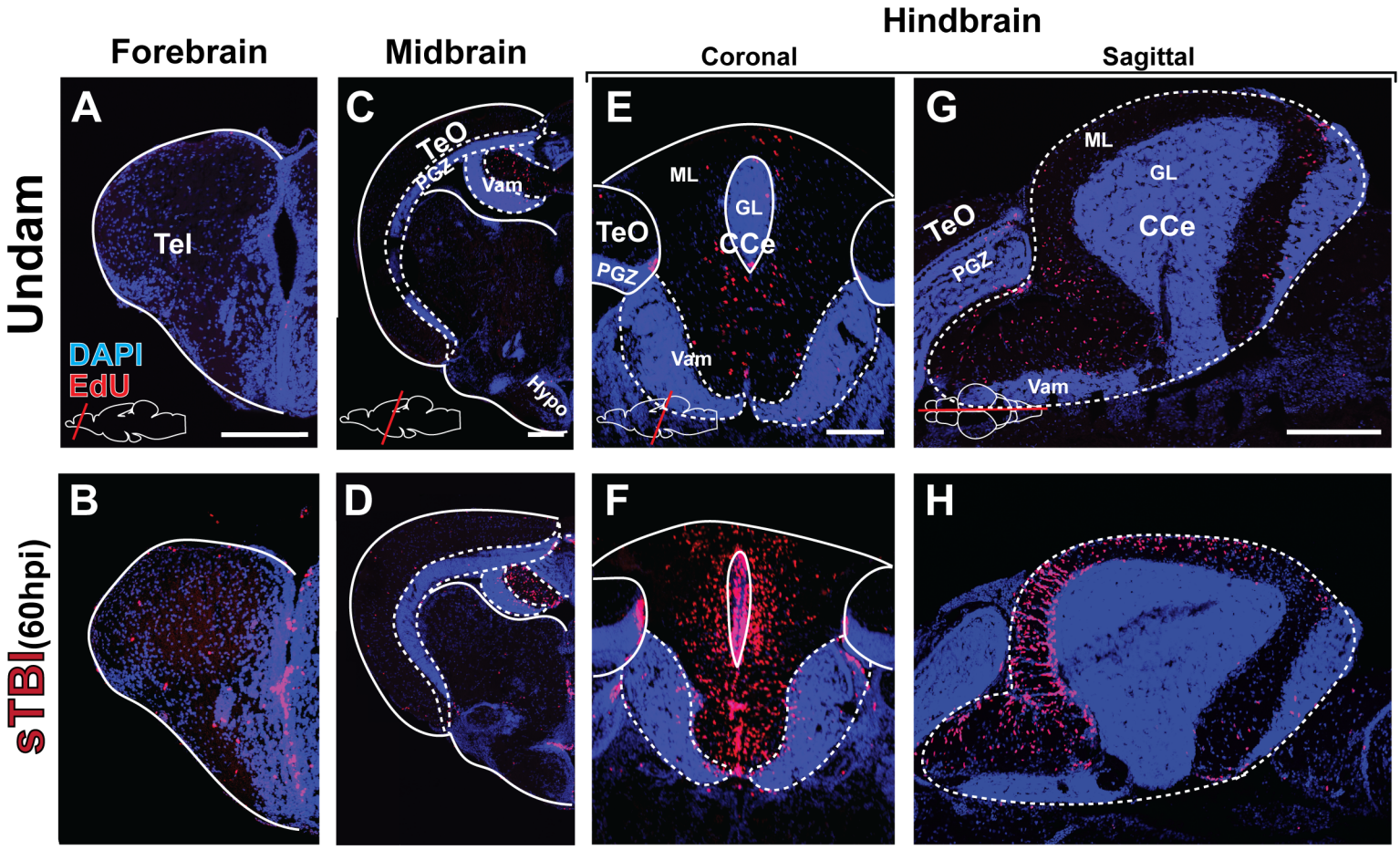

Table 1. Zebrafish display severity-dependent impact

Group	*N*	*n*	Mean Seizures (%) ± SEM	*p*
Undam	10	74	0%	
miTBI	10	100	0%	>0.99
moTBI	10	184	10.66% ± 1.37%	<0.0001
sTBI	10	237	19.93% ± 1.49%	<0.0001

seizures following TBI.

Name of Material/ Equipment	Company	Catalog Number
2-phenoxyethanol	Sigma Alderich	77699
#00 buckshot	Remington	RMS23770
#3 buckshot	Remington	RMS23776
#5 Dumont forceps	WPI	14098
5-ethynyl-2’-deoxyuridine	Life Technologies	A10044
5ml glass vial	VWR	66011-063
Click-iT EdU Cell Proliferation Kit	Life Technologies	C10340
CytoOne 12-well plate	USA Scientific	CC7682-7512
Instant Ocean	Instant Ocean	SS15-10
Super frost postiviely charged slides	VWR	48311-703
Super PAP Pen Liquid Blocker	Ted Pella	22309
Tissue freezing medium	VWR	15148-031

Comments/Description

3.3g weight for sTBI
1.5g weight for miTBI/moTBI

EdU

Below are the comments from the Editors and Reviewers. Under each comment, we describe how we addressed the concern (in bold text).

Editorial comments:

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

We carefully checked the manuscript for spelling and grammatical errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: (Cat#: 77699, Sigma), (Cat#: SS15-10, Instant Ocean), (Super PAP Pen Liquid Blocker, CAT#: 22309, Ted Pella), Click-iT EdU Cell Proliferation Kit (Cat #: 341 C10340, Life Technologies), etc.

All commercial references were removed and confirmed they are present in the materials list.

3. 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."

Each line begins with a verb.

4. The Protocol should contain only action items that direct the reader to do something in complete sentences.

Aspects that are not action items are placed as "Note:…"

5. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Step 1.4.1 has the most action items with 3 actions and notes.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Several points were elaborated.

7. Please include details of how you consider and define mild and severe TBI in this experiment. Citations if any.

Severity is largely defined by impact force and the resultant sequela described in the current manuscript i.e. hematoma formation, seizures, edema, as well as cognitive deficits described in another manuscript that is currently under review at JoVE (JoVE62745). Forthcoming publications are currently not available for citation.

8. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 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.

The main body of the manuscript is 10.5pgs, the protocol is 5.5pgs,and the highlighted content is 2.5pgs.

9. Please ensure the results are described in the context of the presented technique and include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data.

Done

10. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods

e) Any future applications of the technique
We addressed most of these points in the Discussion.

11. 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.
The references were properly formatted.

Reviewer #1:
Manuscript Summary:
Authors modified the commonly used rodent Marmarou weight drop for use in adult zebrafish and established zebrafish blunt-force TBI model. Authors recapitulates many of the phenotypes observed following human TBI, such as contact- and post- traumatic seizures, edema, subdural and intracerebral hematomas, and cognitive impairments, each displayed in an injury severity-dependent manner.
Their zebrafish blunt-force TBI model is novel and promising, and their current technique would allow for investigating disease onset and progression, as well as the mechanisms of neuronal regeneration that is unique to zebrafish. However, as stated in "major points", authors need to clarify the ambiguity of the ingredient of reagents and manipulation process. Only if the authors address all of major points stated below, I recommend this manuscript for publication in JoVE.

Major Concerns:
Line 164: "…create a channel…"
Authors should indicate which part is "channel" in Figure 1.
We labeled the "channel" in the Figure 1 (step 4) and modified the text beginning on Line 152-155 to clarify the creation of the "channel": "*Use a razor blade to divide the raised platform lengthwise into two approximately equal halves (Figure 1, step 3, red dashed line). Form the two halves into a* channel *that accommodates* the length of an adult fish (Figure 1, step 4). Use additional clay to build walls that will secure ~2/3 of the fish body, leaving the head exposed."*

Line 169 1.6: Anesthetize 1 fish…
Do they just put fish in solution or inject drug? What is the final concentration (mg/l ?) of the 2-phenoxyethanol solution? Authors should describe detail manipulation/process for anesthetization using with 2-phenoxyethanol or cite reference.
We modified the anesthesia description on text Line 169-170: "*Anesthetize 1 fish by placing them into a beaker with 50-100 mL of 1:1000 (1mL/1L, 0.1%) 2-phenoxyethanol until it is unresponsive to tail pinch."*

Line 193 1.9: Drop the ball…
Authors should describe that the ball should be drop on which part of fish to cause blunt-force TBI, and indicate it in Figure 1.
Addressed with the following text Line 183-187: "*Drop the ball bearing from a predetermined height for the corresponding desired injury (1.5 g for mild and moderate TBI, and 3.3g for severe TBI) from the top of the tubing onto the steel plate located over the desired neuroanatomical region of interest (e.g. cerebellum, Figure 1, step 7) to produce the blunt-force TBI and place injured fish in a recovery tank to be monitored."*
The corresponding placement of the steel plate is shown in Figure 1, steps 5 and 6, and described on lines 172-175. The ball can be dropped on different regions of the brain to stimulate a blunt-force trauma in different locations.

Line 221 3.1:
What is the final concentration (mg/l ?) of the 2-phenoxyethanol solution? Authors should describe detail.
Addressed with the following text Line 212-213: "*At the desired endpoint, euthanize fish in 1:500 (2ml/1L, 0.2%) solution of 2-phenoxyethanol until gill movements cease and the fish are unreactive to fin pinch."*

Line 227 3.3:
Authors should indicate the location of pin to place in Figure 1.

We feel placing the location of the pins (described explicitly for brain removal after sacrificing the fish) in Figure 1 may confuse the readers and imply fish are pinned during the initial blunt force TBI. Therefore, we prefer not to show the pin placement in Figure 1, unless the Reviewer is completely convinced that this is necessary.

Line 314: …9:1 ethanol:formaldehyde
Is this solution same as described in Line 260 or different? If it is described as "9:1 ethanol:formaldehyde", this means 90% EtOH, 10% formaldehyde. But one in Line 260 is 90% EtOH, 3.7% formaldehyde. Authors should clarify this point.
Both lines (Lines 251-252 and 303-306) have been addressed to clarify. "*Fix the removed brains in 9 parts 100% ethanol to 1 part 37% formaldehyde overnight at 4˚C on a rocker platform."*

Line 325: …storing them in 2:1 solution of tissue freezing medium (TFM, Cat #: 15148-031, VWR International) and 30% sucrose/PBS overnight at 4 ˚C on a rocker platform.
The final ingredient of storing solution is not clear. "2:1 solution" and "30% sucrose/PBS" are different solution or same solution? If it is different, what is time length of the incubation of "2:1 solution"?
Addressed with the following text Line 313-316: "*Remove brains from 30% sucrose/PBS and transfer them with forceps into a 12-well plate (1 treatment group per well) with wells filled with a 2:1 solution consisting of 2 parts tissue freezing medium and 1 part 30% sucrose/PBS. Incubate the brains overnight at 4˚ C on a rocker platform."*

Minor Concerns:
Line 124:
KE=1/2mv2 should be KE=(1/2)mv2 ? It is confusing with 1 /(2m mv2)
Because we removed the calculation of velocity (see Reviewer #2, minor concern #1), we used the following equation for calculating kinetic energy: $KE = mgh$***. Lines 122-126 now describe this equally accurate and more straightforward equation.***

Line 353-354:
It is recommended that authors elucidate abbreviation; miTBI (mild TBI), moTBI (moderate TBI), and sTBI (severe TBI).
Addressed in the protocol upon the introduction of each severity level.

Reviewer #2:
Manuscript Summary:
Due to its unique ability to regenerate neurons of the central nervous system upon injury, the adult zebrafish is a very interesting and relevant model to study brain injury. In this manuscript, the authors provide a well-documented extension of a standard assay for traumatic blunt-force brain injuries to the zebrafish model and discuss multiple potential applications in future research towards identifying the causes for this remarkable regenerative capability. Using their assay, the authors identify a previously undescribed, graded response in brain cell proliferation upon injuries of graded severity. The authors comply with animal welfare regulations throughout. Images and text are informative and clear and will be a helpful resource in JoVE together with video instructions.

Major Concerns:
none

Minor Concerns:
lines 121-161: The expression for velocity as $v = \sqrt{2gh}$ comes from equating loss in potential energy mgh to increase in kinetic energy: mgh = 1/2 mv^2 and solving for v. Since velocity is never measured but only inferred from drop height, it seems redundant to first take the square root to calculate the velocity as a numerical value, then squaring it again to calculate kinetic energy. Simpler would be to make a statement about energy conservation and use mgh to calculate energy as well as force, and give the velocity information in an extra statement for interest.
The reviewer is correct. We removed the calculation of velocity from the text and gave the direct

equation for calculating kinetic energy. We also remove the calculation of impact force as it is based on the distance the object (skull or the plate on the skull) moves after being struck. Because this is difficult to determine without highspeed video, we removed it from the discussion and only show the calculation of kinetic energy.

line 121: either draw the square root across the whole expression mgh, or put (2gh) in parentheses, to avoid reading $\sqrt{2} \times g \times h$.
This calculation was removed when we removed the calculation of velocity (see above).

line 130: mass, add "(in kg)" to match the other SI units given.
We made the suggested change on Line 124

line 308: µl instead of ul
We made the appropriate change.

Fig. 2C: the red cross at the right parietal plate suggests that one should not use this access point, whereas the arrow and text suggests that one should take it there to remove it - a less prohibitive sign and color for highlighting the access point is recommended.
We removed the red cross and placed an arrow in its place.

Table 1 and Table 2: Box plots would be valuable visuals instead of only giving mean and SEM in a table.
We placed the Edema data into a Box plot (new Figure 4). However, we retained the seizure data in Table 1 to better represent the number of trials and fish that were analyzed in each damage severity. We felt that this information would be lost in a box plot

Legends to Table 1 and Table 2: What N and n are should be stated in the legend.
We defined N and n in the appropriate legends.

Legends to Table 1 and Table 2: A normal Tukey post-hoc test requires identical group sizes, which is not the case here; the Tukey-Kramer test corrects for unequal sample sizes, the Games-Howell-Test in addition for heterogeneous variances; a corresponding test should be used and the exact name stated.
For the Seizure data (Table 1), we increased the number of experimental groups (N) so that all the severities have an N of 10, which allowed us to perform a One-way ANOVA and a Tukey's post-hoc test. This is stated in the Table legend.

For the Edema data (Figure 4), A Browns-Forsythe and Welch ANOVA was used with a Dunnett's T3 multiple comparison post-hoc test. By using Browns-Forsythe and Welch ANOVA we do not assume equal SD. The reviewer suggested a Games-Howell post-hoc test, however, this post-hoc test is for n>50 and our software Prism (GraphPad) 9.0 suggested a Dunnett's T3 post-hoc as each group is n<50. The statistical conclusions remain the same across both post-hoc exams.

Statistical software should be given.
We performed all statistical analyses with the Prism (Graphpad 9.0) software package and this is now stated in the appropriate legends.