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Scriptwriter Name: Nilesh Kolhe
Supervisor Name: Anastasia Gomez
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Title: Shuttle Box Assay as an Associative Learning Tool for Cognitive Assessment in Learning and Memory Studies using Adult Zebrafish

Authors and Affiliations: James Hentig[1,2,3], Kaylee Cloghessy[1,2,3], David R. Hyde[1,2,3]

[1]Department of Biological Sciences, University of Notre Dame, Notre Dame, USA
[2]Center for Zebrafish Research, University of Notre Dame, Notre Dame, USA
[3]Center for Stem Cells and Regenerative Medicine, University of Notre Dame, Notre Dame, USA

Corresponding Authors:

David R. Hyde dhyde@nd.edu

Email Addresses for All Authors:
jhentig@nd.edu
kcloghes@nd.edu
dhyde@nd.edu

Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group?

   Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **NO**

Current Protocol Length

Number of Steps: 20
Number of Shots: 43

Introduction

1. Introductory Interview Statements

1.1. **David Hyde:** The shuttle box assay reproducibly tracks the progression and recovery of cognitive impairment following a blunt-force trauma or any other type of brain injury in zebrafish **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **David Hyde:** The shuttle box assay allows for relatively simple, rapid, and robust measurement of associative learning and both short and long-term memory in zebrafish **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.7, 3.8, 3.10 and 3.11*

Introduction of Demonstrator on Camera

1.3. **David Hyde:** Demonstrating the procedure will be Mr. Jayme Hentig and Ms. Kaylee Cloghessy, two doctoral students from my laboratory **[1] [2]**.

1.3.1. INTERVIEW: Named talent saying the above.

1.3.2. The named demonstrator(s) looks up from the workbench or desk, or microscope and acknowledges the camera.

Ethics Title Card

1.4. Tissue collection and processing of human cancer specimens were performed in compliance with the NHS Confidentiality Code of Practice and with the Data Protection Act 1998 of the University Hospitals of Leicester NHS Trust policy and procedure.

Protocol

2. Shuttle Box Learning Paradigm

2.1. To begin, prepare the shuttle box by modifying a 30.5 by 19 by 7.5-centimeter gel box with a 5 by 19-centimeter piece of aquarium grade plexiglass added to each side at a 45-degree angle **[1]**, then make a line marking the halfway point of the tank to assess when fish have crossed the middle of the tank **[2]**.

2.1.1. WIDE: Establishing shot of talent preparing the shuttle box of appropriate dimensions

2.1.2. Talent making a line mark to the halfway point of the tank

2.2. After adding 800 milliliters of system water to the shuttle box **[1]**, place 2-3 fish into a holding tank containing system water, located in a dark room where the shuttle box assay will be performed **[2]**.

2.2.1. Talent adding system water to the shuttle box

2.2.2. Talent placing fish into a holding tank

2.3. Next, in the dark room, place one fish in the center of the shuttle box **[1]**, secure the lid **[2],** and attach the electrodes to a power supply **[3]**.

2.3.1. Talent placing fish in the center of the shuttle box

2.3.2. Talent locking/securing the lid

2.3.3. Talent attaching the electrodes to a power supply

2.4. Acclimate the fish in the shuttle box for 15 minutes **[1]**. Successful acclimation can be considered when the fish freely explores the tank **[2-TXT]**.

2.4.1. Talent leaving the fish for acclimation

2.4.2. Shot of fish freely exploring the tank **TEXT: Refer to the text if fish fails to explore**

2.5. After successful acclimation of the fish, manually shine an 800-lumen red lens flashlight approximately 2 centimeters from the gel box wall on the side occupied by the fish. Do not start a trial if the fish is resting next to the platinum wire **[1]**.

2.5.1. Talent shining red lens flashlight on the side occupied by the fish.

NOTE: Shots 2.5.1., 2.6.1. and 2.6.2. were all combined into a single shot.

2.6. Shine the light stimulus directly on the fish **[1]** and manually follow any lateral movement of the fish with the light to ensure continual visualization of the stimulus. Continue to provide the light stimulus until either of the following conditions for a successful or failed trial are met **[2].** *Videographer: This step is important!*

2.6.1. Talent shining the light stimulus on the fish

2.6.2. Shot of lateral movement of the fish

NOTE: Shots 2.5.1., 2.6.1. and 2.6.2. were all combined into a single shot and the appropriate steps were called out during filming.

2.7. Consider the trial successful if the fish crosses over the halfway point of the tank within 15 seconds of light exposure **[1]**. Once the fish crosses the halfway point, stop the light stimulus immediately **[2]**. *Videographer: This step is important!*

2.7.1. Shot of fish crossing over the halfway point of the tank

2.7.2. Talent stopping the light stimulus

NOTE: The trials of the fish which include shots 2.7.1. ,2.7.1. ,2.8.1. and 2.8.2. were all shot together, and the appropriate step number was announced during filming for each of the multiple sequences filmed in a singular shot.

2.8. If the trial failed, use an electrophoresis power supply to apply a negative shock stimulus of 20 millivolts to 1 Ampere alternating 2 seconds On, 2 seconds Off for 15 seconds with a maximum of 4 shocks, or until the fish passes the halfway point of the box **[1]**. Then, terminate both the light and negative stimulus **[2]**. *Videographer: This step is important!*

2.8.1. Talent using electrophoresis power supply to apply a negative shock stimulus

2.8.2. Talent terminating both the light and negative stimulus

3. Memory Paradigm

3.1. During the training period, perform a successful acclimation in the shuttle box for 15 minutes **[1]**, then manually shine an 800-lumen red lens flashlight approximately 2 centimeters from the gel box wall on the side occupied by the fish **[2]**.

3.1.1. Shot of fish freely exploring the tank

3.1.2. Talent shining red lens flashlight on the side occupied by the fish

3.2. Shine the light stimulus directly on the fish **[1]** and follow any lateral movement of the fish with the light to ensure continual visualization of the stimulus **[2].**

3.2.1. Talent shining the light stimulus on the fish

3.2.2. Shot of lateral movement of the fish

3.3. While the light is shining on the fish **[1]**, simultaneously apply the adverse shock stimulus of 20 millivolts to 1 Ampere alternating 2 seconds On, 2 seconds Off for 15 seconds with a maximum of 4 shocks or until the fish passes the halfway point of the box **[2]**. Once this is achieved, terminate both the light and adverse stimulus **[3]**. *Videographer: This step is important!*

3.3.1. Shot of light shining on the fish

3.3.2. Talent applying adverse shock stimulus

3.3.3. Talent terminating both the light and adverse stimulus

3.4. During the Initial Testing Period, perform a successful acclimatization in the shuttle box for 15 minutes, then apply only the light stimulus for up to 15 seconds **[1]** and record the responses **[2]**. *Videographer: This step is important!*

3.4.1. Talent applying the light stimulus

3.4.2. Talent recording the responses

3.5. Consider the trial successful if the fish crosses over the halfway point of the shuttle box within 15 seconds after starting the light stimulus **[1]** and stop the light stimulus immediately when the fish crosses the halfway point **[2]**.

3.5.1. Shot of fish crossing over the halfway point of the tank

3.5.2. Talent stopping the light stimulus

NOTE: Shots 3.5.1. , 3.8.2. and 3.11.2. represent the same process and can be used interchangeably.

3.6. Consider the trial as failed if the fish does not cross over the halfway point of the shuttle box 15 seconds after starting the light stimulus **[1]** and stop the light stimulus after 15 seconds. During the initial testing, do not apply an adverse stimulus following a failed attempt **[2]**.

3.6.1. Shot of fish not crossing over the halfway point of the tank/failed trial

3.6.2. Talent stopping the light stimulus

NOTE: Shots 3.6.1. , 3.8.3. and 3.11.3. represent the same process and can be used interchangeably.

NOTE: For shot 3.6.1. the response of the fish was filmed over period of time and the author has then mentioned in the video if the trial was positive (3.5.1.) or negative (3.6.1.).

3.7. Perform Short-Term Memory Testing immediately following the initial testing period. Induce traumatic brain injury for four hours **[1],** then acclimate the fish in the shuttle box for 15 minutes **[2]**.

3.7.1. Talent inducing brain injury to the fish

3.7.2. Talent leaving the fish for acclimation

3.8. Assess short-term memory by applying only the light stimulus for up to 15 seconds **[1]** and record if the fish crosses over the halfway point of the box before the light is turned off, considered as pass trial **[2]** or fails to cross the halfway point within the 15 seconds, considered as failed trial **[3]**.

3.8.1. Talent applying the light stimulus

3.8.2. Shot of fish crossing over the halfway point of the tank/passed trial

3.8.3. Shot of fish not crossing over the halfway point of the tank/failed trial

NOTE: Shots 3.5.1. , 3.8.2. and 3.11.2. represent the same process and can be used interchangeably.

NOTE: Shots 3.6.1. , 3.8.3. and 3.11.3. represent the same process and can be used interchangeably.

3.9. Repeat the above step 25 times with a 30 second rest period between each trial **[1]** and record the number of successful and failed trials **[2]**.

3.9.1. Talent applying the light stimulus

3.9.2. Talent recording the responses of successful and failed trials

3.10. Perform Long-Term Memory Testing 4 days after initial testing. Induce traumatic brain injury for four hours **[1],** then acclimate the fish in the shuttle box for 15 minutes **[2]**.

3.10.1. Talent inducing brain injury to the fish

3.10.2. Talent leaving the fish for acclimation

3.11. Assess long-term memory by applying only the light stimulus for up to 15 seconds **[1]** and record if the fish crosses over the halfway point of the box before the light is turned off, considered as pass trial **[2]** or fails to cross the halfway point within the 15 seconds, considered as failed trial **[3]**.

3.11.1. Talent applying the light stimulus

3.11.2. Shot of fish crossing over the halfway point of the tank/passed trial

3.11.3. Shot of fish not crossing over the halfway point of the tank/failed trial

NOTE: Shots 3.5.1. , 3.8.2. and 3.11.2. represent the same process and can be used interchangeably.

NOTE: Shots 3.6.1. , 3.8.3. and 3.11.3. represent the same process and can be used interchangeably.

3.12. Repeat the above 25 times with a 30 second rest period between each trial **[1]** and record the number of successful and failed trials **[2]**.

3.12.1. Talent applying the light stimulus

3.12.2. Talent recording the responses of successful and failed trials

Results

4. Results: Shuttle Box Assay as an Associative Learning Tool for Cognitive Assessment in Learning and Memory Studies using Adult Zebrafish

4.1. The instructional overview of the learning **[1]** and memory paradigms for cognitive assessment is shown here **[2]**.
- 4.1.1. LAB MEDIA: Figure 1 *Video Editor: please emphasize on learning paradigm from the figure*
- 4.1.2. LAB MEDIA: Figure 1A *Video Editor: please emphasize on memory paradigm from the figure*

4.2. The undamaged fish at 8 months, young adult, 18 months, middle-aged adult, and 24 months, elderly adult required a similar number of trials to learn the behavior of avoiding the red light **[1]**.
- 4.2.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize on Undam from 8 months, 18 months, and 24 months graph*

4.3. After utilizing the severe blunt-force traumatic brain injury model, or sTBI *(Severe-T-B-I)*, the fish at different ages required a similar number of trials to master the assay across 1 to 5 days post-injury **[1]**.
- 4.3.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize on 1 dpi, 2 dpi, 3 dpi, 4 dpi, and 5 dpi each from 8 months, 18 months, and 24 months graph*

4.4. On Day 1 following sTBI, fish of all ages required a similar number of trials to learn the behavior **[1]**, which was significantly greater than the undamaged controls **[2]**.
- 4.4.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize on 1 dpi from 8 months, 18 months, and 24 months graph*
- 4.4.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize on Undam from 8 months, 18 months, and 24 months graph*

4.5. The undamaged fish rapidly mastered the shuttle-box, achieving 5 consecutive positive trials in approximately 17 trials **[1]**. Whereas, one day following a mild brain injury, or miTBI *(Mild-T-B-I)*, fish display a significant increase in the number of trials to learn the behavior **[2]**.
- 4.5.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize on Undam bar from the graph*
- 4.5.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize on 1x bars from the graph*

4.6. This deficit increased after 2 miTBI **[1]** and was further elevated after 3 miTBI injuries **[2]**.
  4.6.1. LAB MEDIA: Figure 2B *Video Editor: please emphasize on 2x bar from the graph*
  4.6.2. LAB MEDIA: Figure 2B *Video Editor: please emphasize on 2x bar from the graph*

4.7. Undamaged fish exhibit a slight increase in the percent difference of successful trials in immediate memory and delayed memory relative to the initial testing period **[1]**.
  4.7.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize Undam from the graph*

4.8. Following a single miTBI, fish displayed significant and immediate memory deficits **[1]** compared to undamaged fish **[2]**. This trend continued with repeated injury with increasing deficits following both 2 miTBI **[3]** and 3 miTBI **[4]**.
  4.8.1. LAB MEDIA: Figure 2C *Video Editor: please emphasize on 1x from the graph*
  4.8.2. LAB MEDIA: Figure 2C *Video Editor: please emphasize on undam from the graph*
  4.8.3. LAB MEDIA: Figure 2C *Video Editor: please emphasize on both 2x miTBI from the graph*
  4.8.4. LAB MEDIA: Figure 2C *Video Editor: please emphasize on both 3x miTBI from the graph*

Conclusion

5. Conclusion Interview Statements

5.1. **Kaylee Cloghessy:** The timing of applied adverse stimuli is critical. Pairing the adverse stimuli with the light and their simultaneous removal solidifies their association and is crucial for the paradigm **[1]**.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.8, 3.3, 3.5 and 3.6*

5.2. **James Hentig:** This assay allows for rapid assessment of complex associative learning that could be used to investigate developmental, aging, and environmental impacts on cognitive impairment **[1]**.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.