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Title: Low-Cost Alternative for Examining Social Preference in Zebrafish (*Danio rerio*) Using the 3-Chamber Open-Tank Free-Swim Task

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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? **Yes, all done**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? 5-Minute walk, approximately .3 miles.

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **Yes.**

Current Protocol Length

Number of Steps: 23

Number of Shots: 58 (45 SC)

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Mikaela Scott:** This protocol validates a low-cost and open-source, three-chamber open-tank free swim task investigating social preference in zebrafish.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Describe the research contexts or systems in which this protocol can be applied.**~~

- 1.2. **Kaitlyn Kinslow:** This protocol can be used with various species and allows undergraduate researchers to investigate social preference with minimal resources.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.3. **Mikaela Scott:** This protocol can be used by researchers to investigate a zebrafish's tendency to visually engage with two different social stimuli and determine which stimulus is preferred.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~**Are there any additional methods or analyses that can be performed following this procedure?**~~

- 1.4. **Kaitlyn Kinslow:** Researchers can use different species or stimulus configurations as well as investigate other responses such as darting or erratic swimming.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Mikaela Scott:** The system offers a platform to study translational models for social functionality and could explore pharmacological and environmental manipulations.
- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Testimonial Questions (OPTIONAL):

Videographer: Please capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your JoVE article address?

1.6. **Kaitlyn Kinslow, Undergraduate Researcher – Christopher Newport University**

: (authors will present their testimonial statements live)

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

What is the key takeaway you hope viewers remember after watching your video article?

1.7. **Mikaela Scott, High School Researcher – Governor's School for Science & Technology**: (authors will present their testimonial statements live)

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

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at the Christopher Newport University

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. OBS Studio Configuration for Zebrafish Behavioral Video Acquisition

Demonstrators: Mikaela Scott and Kaitlyn Kinslow

2.1. To begin, prepare the open tank apparatus [1] with a central experimental chamber and 2 flanking stimulus chambers [2]. Install the backlighting system, opaque dividers, and polarizing filters to optimize visual isolation and video contrast [3]. Mount and align the cameras in front of the chambers using the camera support frame [4]. Prepare the testing environment with blackout curtains and stable holding conditions before initiating behavioral testing [5].

- 2.1.1. WIDE: Talent showing the complete 3-chamber open-tank free-swim task apparatus.
- 2.1.2. Close-up of the apparatus and pointing to the central experimental chamber and 2 flanking stimulus chambers.
- 2.1.3. Talent positioning or adjusting the positions of the backlighting system, opaque dividers, and polarizing filters around the chambers.
- 2.1.4. Talent mounting the camera on the camera support frame in front of the chambers.
- 2.1.5. Talent drawing the blackout curtains in the testing area.

2.2. Download the **Open Broadcaster Software** and install it on a Windows-operated local computer [1]. After launching the software, run the **Auto-Configuration Wizard** to optimize the settings for the specific recording needs, hardware resources, and network conditions [2].

2.2.1. SCREEN: 70610_2.2.1-(1).mp4.

2.2.2. SCREEN: 70610_2.2.3.mp4.

2.3. Verify the basic settings before camera configuration [1]. Set the **Base (Canvas) Resolution** (*base canvas resolution*) to match the display resolution [2] and set the appropriate **Output (Scaled) Resolution** (*output scaled resolution*) [3].

2.3.1. SCREEN: 70610_2.3.1-2.3.3.mp4 00:00-00:04.

2.3.2. SCREEN: 70610_2.3.1-2.3.3.mp4 00:05-00:07.

2.3.3. SCREEN: 70610_2.3.1-2.3.3.mp4 00:08-00:12.

- 2.4. Then, create a scene by right-clicking in the **Scenes** box and selecting **Add scene [1]**. Assign a name to the scene and use it to define the stream layout **[2]**.

2.4.1. SCREEN: 70610_2.4.1-2.4.3.mp4. 00:00-00:04

2.4.2. SCREEN: 70610_2.4.1-2.4.3.mp4. 00:04-00:12

- 2.5. Add a new source **[1]** and select **Video Capture Device** to add a webcam **[2]**. Assign each camera a distinct name, such as **camera-center** and repeat the process for the additional camera **[3]**.

2.5.1. SCREEN: 70610_2.5.1-2.5.3.mp4. 00:00-00:02

2.5.2. SCREEN: 70610_2.5.1-2.5.3.mp4. 00:03-00:07.

2.5.3. SCREEN: 70610_2.5.1-2.5.3.mp4. 00:08-00:23.

- ~~2.6. Repeat the process for additional cameras or sources as needed **[1]**.~~

~~2.6.1. SCREEN: Additional cameras or sources being added.~~

- 2.7. Position and resize the sources in the preview window **[1]**. Ensure that the camera is listed in the **Sources** list **[2]**.

2.7.1. SCREEN: 70610_2.7.1-2.7.2.mp4 00:00-00:15.

2.7.2. SCREEN: 70610_2.7.1-2.7.2.mp4 00:18-00:23.

- 2.8. Bring the camera view to the center of the canvas by pressing **Control+D** (*control plus D*) **[1]**.

2.8.1. SCREEN: 70610_2.7.1-2.8.2.mp4.

- 2.9. Apply Fit to Screen by pressing **Control+F** (*control plus F*) **[1]** or stretch the screen by pressing **Control+S** (*control plus S*) **[2]**. Crop the source by holding the **Alt** key while dragging the bounding box **[3]**.

2.9.1. SCREEN: 70610_2.9.1-2.9.3.mp4 00:04-00:08.

2.9.2. SCREEN: 70610_2.9.1-2.9.3.mp4 00:09-00:15.

2.9.3. SCREEN: 70610_2.9.1-2.9.3.mp4 00:20-00:27.

2.10. Next, double-click on the selected camera and click on **Configure Video [1]**. Adjust the video's brightness and contrast in the Properties menu **[2]**.

2.10.1. SCREEN: 70610_2.10.1-2.10.3.mp4 00:00-00:08.

2.10.2. SCREEN: 70610_2.10.1-2.10.3.mp4 00:10-00:19.

3. Acclimation of Adult Zebrafish in the 3-Chamber Open-Tank

Demonstrator: Kaitlyn Kinslow

3.1. Fill all 3 chambers with water from the housing tanks **[1]**. Place opaque barriers between the chambers and leave them in place throughout the acclimation period **[2]**. Transfer 1 adult *Danio rerio* to the central chamber and allow it to acclimate for at least 1 hour **[3]**.

3.1.1. Talent filling all 3 chambers with water from the housing tanks.

3.1.2. Talent placing opaque barriers between the chambers.

3.1.3. Talent transferring 1 zebrafish to the center experimental chamber.

3.2. Place the artificial social stimuli in the flanking left stimulus chamber **[2]**. Then, transfer a small shoal of 4 fish, consisting of 2 males and 2 females of similar size, age, and familiarity, to the right stimulus chamber **[3]**.

3.2.1. Talent placing the artificial social stimuli in the left stimulus chamber.

3.2.2. Talent transferring the shoal of 4 fish to the right stimulus chamber.

3.3. During acclimation, supply all chambers containing live animals with a submersible heater set at 26 degrees Celsius, a bubbler stone connected to the aquarium air pump, and a tank lid to maintain stable holding conditions **[1]**.

3.3.1. Talent placing the submersible heater, bubbler stone connected to the aquarium air pump, and tank lid on all chambers containing live animals.

4. Experimental Social Preference Testing

- 4.1. Remove the heaters and bubbler stones before initiating the experimental session [2].
 - 4.1.1. Talent removing the heaters and bubbler stones before initiating the experimental session.
- 4.2. ~~Place the social stimuli being tested in each flanking tank [1].~~ Transfer the test subject to the center tank [2]. Start the single camera on the center tank to record video of the test subject [3].
 - 4.2.1. ~~WIDE: Talent placing the social stimuli in each flanking tank.~~
 - 4.2.2. ~~Talent transferring the test subject to the center tank.~~
 - 4.2.3. Talent starting the camera and Shot of the single camera on the center tank recording video for the test subject.
- 4.3. Launch the software and resize the central camera view to fill the screen by clicking and dragging the video corners [1]. Then, select **Start Recording** under **Controls** [2].
 - 4.3.1. SCREEN: 70610_4.3.1-4.3.2.mp4 00:00-00:10.
 - 4.3.2. SCREEN: 70610_4.3.1-4.3.2.mp4 00:11-00:17.
- 4.4. Immediately after recording is initiated, remove the opaque barriers using the pulley-string system and continue recording for 10 minutes [1].
 - 4.4.1. Talent removing the opaque barriers using the pulley-string system and observing the tank/pointing to the freely moving zebrafish in the tank.
- 4.5. End the session by selecting **Stop Recording** under **Controls** [1]. Access the recorded sessions by selecting **File** in the top-left menu and choosing **Show Recordings** from the drop-down menu [2].
 - 4.5.1. SCREEN: 70610_4.5.1-4.5.2.mp4 00:00-00:03.
 - 4.5.2. SCREEN: 70610_4.5.1-4.5.2.mp4 00:04-00:14.

5. Motion Tracking and Behavioral Analysis of Zebrafish Social Preference

- 5.1. Load 4 to 5 videos into a motion-analysis software application, such as DeepLabCut [1] and extract 20 to 30 frames from each video [2]. Define at least 3 primary tracking points, such as the head, tail and trunk. Train the network to track these points across the frames [3].

5.1.1. SCREEN: 70610_5.1.1.mp4 00:32-0:42.

5.1.2. SCREEN: 70610_5.1.2.mp4. 00:23-00:29

5.1.3. SCREEN: 70610_5.1.3-5.1.4.mp4 00:12-00:26.

- 5.2. Use a ResNet-101-based neural network to train the model [1]. Determine the number of iterations required to complete training [2-TXT].

5.2.1. SCREEN: 70610_5.2.1-5.2.3.mp4 00:00-00:06.

5.2.2. SCREEN: 70610_5.2.1-5.2.3.mp4 00:16-00:21 and 00:26-00:28. **TXT: P-cutoff value: 0.5 - 1**

- 5.3. Next, deploy the fully trained DeepLabCut model on the remaining experimental videos [1] and extract positional data for each trial, following the user guide [2-TXT].

5.3.1. SCREEN: 70610_5.3.1.mp4. 00:03-00:10 and 00:18-00:20

5.3.2. SCREEN: 70610_5.3.2.mp4.

TXT: <https://deeplabcut.github.io/DeepLabCut/docs/beginner-guides/Training-Evaluation.html>

- 5.4. Then, use a coding software, such as Python, to define and analyze movement across the 3 vertical arena zones of equal width to quantify preference [1]. Measure the time in each zone using at least 2 body points within the identified zone to reduce tracking noise [2]. Calculate the total time in each zone by dividing the total frames in the zone by the total frame count [3].

5.4.1. SCREEN: 70610_5.4.1-5.4.3.mp4 00:00-00:05 and 00:12-00:14.

5.4.2. SCREEN: 70610_5.4.1-5.4.3.mp4 00:18-00:20.

5.4.3. SCREEN: 70610_5.4.1-5.4.3.mp4 00:30-00:35 and 00:50-00:52.

- 5.5. Use the same coding software to define and measure the time spent motionless, such as freezing [1]. Define the movement threshold at 2 to 5 pixels per frame with a minimum consecutive frame count of 15 frames [2].

5.5.1. SCREEN: 70610_5.5.1-5.5.2.mp4 00:00-00:10.

5.5.2. SCREEN: 70610_5.5.1-5.5.2.mp4 00:22-00:30.

5.6. Finally, compile and export the measurements for statistical analysis and visualization [1].

5.6.1. SCREEN: 70610_5.5.1-5.5.2.mp4 00:03-00:05 and 00:15-00:20.

Results

6. Results

6.1. Subjects spent significantly more session time in the right zone closest to the live shoal [1-TXT] than in the middle zone [2] or the left zone closest to the artificial mobile stimulus [3]. The difference in time spent between the middle zone and the left zone was not statistically significant [4].

6.1.1. LAB MEDIA: Figure 3A. *Video editor: Highlight the bar labeled Right-Live Shoal. TXT: (N = 23, 11 males and 12 females)*

6.1.2. LAB MEDIA: Figure 3A. *Video editor: Highlight the bar labeled Middle.*

6.1.3. LAB MEDIA: Figure 3A. *Video editor: Highlight the bar labeled Left-Mobile.*

6.1.4. LAB MEDIA: Figure 3A. *Video editor: Highlight the comparison line between Middle and Left-Mobile labeled (ns).*

6.2. Subjects spent significantly less session time motionless in the middle zone [1] than in the right zone closest to the live shoal [2] or the left zone closest to the mobile [3].

6.2.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the bar labeled Middle.*

6.2.2. LAB MEDIA: Figure 3B. *Video editor: Highlight the bar labeled Right-Live Shoal.*

6.2.3. LAB MEDIA: Figure 3B. *Video editor: Highlight the bar labeled Left-Mobile.*

1. Zebrafish

Pronunciation link: <https://www.merriam-webster.com/dictionary/zebrafish>

IPA: /'zi:brəˌfɪʃ/

Phonetic Spelling: zee·bruh·fish

2. Behavioral

Pronunciation link: <https://www.merriam-webster.com/dictionary/behavioral>

IPA: /bɪˈheɪvjərəl/

Phonetic Spelling: bih·hayv·yuh·ruhl

3. Apparatus

Pronunciation link: <https://www.merriam-webster.com/dictionary/apparatus>

IPA: /ˌæpəˈrætəs/

Phonetic Spelling: ap·uh·rat·uhs

4. Experimental

Pronunciation link: <https://www.merriam-webster.com/dictionary/experimental>

IPA: /ɪkˌsperəˈmentəl/

Phonetic Spelling: ik·speh·ruh·men·tuhl

5. Stimulus
Pronunciation link: <https://www.merriam-webster.com/dictionary/stimulus>
IPA: /'stimjələs/
Phonetic Spelling: stim·yuh·luhs
6. Polarizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/polarizing>
IPA: /'pəʊləˌraɪzɪŋ/
Phonetic Spelling: poh·luh·rye·zing
7. Acclimation
Pronunciation link: <https://www.merriam-webster.com/dictionary/acclimation>
IPA: /ˌæklə'meɪʃən/
Phonetic Spelling: ak·luh·may·shuhn
8. Danio
Pronunciation link: <https://www.merriam-webster.com/dictionary/danio>
IPA: /'deɪniəʊ/
Phonetic Spelling: day·nee·oh
9. rerio
Pronunciation link: <https://www.howtopronounce.com/rerio>
IPA: /'rɪriəʊ/
Phonetic Spelling: ree·ree·oh
10. Submersible
Pronunciation link: <https://www.merriam-webster.com/dictionary/submersible>
IPA: /səb'mɜːsəbəl/
Phonetic Spelling: sub·mur·suh·buhl
11. Contrast
Pronunciation link: <https://www.merriam-webster.com/dictionary/contrast>
IPA: /'kənˌtræst/, /kən'træst/
Phonetic Spelling: kon·trast; kuhn·trast
12. Resolution
Pronunciation link: <https://www.merriam-webster.com/dictionary/resolution>
IPA: /ˌrezə'luːʃən/
Phonetic Spelling: rez·uh·loo·shuhn
13. DeepLabCut
Pronunciation link: <https://www.howtopronounce.com/deeplabcut>
IPA: /'diːp læb kʌt/
Phonetic Spelling: deep·lab·cut
14. Neural
Pronunciation link: <https://www.merriam-webster.com/dictionary/neural>
IPA: /'nɒrəl/, /'njʊrəl/
Phonetic Spelling: noor·uhl; nyoor·uhl
15. Positional
Pronunciation link: <https://www.merriam-webster.com/dictionary/positional>
IPA: /pə'zɪʃənəl/
Phonetic Spelling: puh·zish·uh·nuhl

16. Statistical

Pronunciation link: <https://www.merriam-webster.com/dictionary/statistical>

IPA: /stə'tɪstɪkəl/

Phonetic Spelling: stuh·tis·ti·kuhl

17. Visualization

Pronunciation link: <https://www.merriam-webster.com/dictionary/visualization>

IPA: /ˌvɪʒuələ'zeɪʃən/

Phonetic Spelling: vɪzh·oo·uh·luh·zay·shuhn

18. Motionless

Pronunciation link: <https://www.merriam-webster.com/dictionary/motionless>

IPA: /'moʊʃənləs/

Phonetic Spelling: moh·shuhn·luhs

19. Consecutive

Pronunciation link: <https://www.merriam-webster.com/dictionary/consecutive>

IPA: /kən'sekjətɪv/

Phonetic Spelling: kuhn·sek·yuh·tiv

20. Configuration

Pronunciation link: <https://www.merriam-webster.com/dictionary/configuration>

IPA: /kənˌfɪɡjə'reɪʃən/

Phonetic Spelling: kuhn·fig·yuh·ray·shuhn