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Title: Phenotypic Profiling of MPTP-Induced Parkinsonian-like Behavioral Phenotypes in Zebrafish Larvae Based on Behavioral Experiment

Authors and Affiliations:

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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? **Yes**

SCOPE: 2.8.1-2.8.2

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

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

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. **No**

Current Protocol Length

Number of Steps: 24

Number of Shots: 52

Introduction

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. **Xiang Li:** We present a multiparametric behavioral protocol to comprehensively profile motor and non-motor Parkinsonian phenotypes in larval 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. **Zhuang Miao:** This protocol applies to Parkinson's disease modeling, drug screening, and environmental neurotoxicity studies using zebrafish larvae.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Zhuang Miao:** The key challenge is minimizing external disturbances while accounting for individual variability to ensure accurate and reproducible behavioral analysis in zebrafish larvae.
 - 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. **Jiying Bi**: Behavioral clustering and phenomic profiling can be applied to uncover subtle neurotoxic effects and gain deeper mechanistic insights.

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. **Jiying Bi**: Future studies can apply this platform to high-throughput drug screening and pharmacological evaluation in zebrafish Parkinson's disease models.

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.

Ethics Title Card

This research has been approved by the Ethical Committee of the School of Pharmaceutical Sciences, Shandong University

Protocol

2. Zebrafish Embryo Preparation for Behavioral Assessment

Demonstrator: Zhuang Miao

- 2.1. To begin, wear appropriate personal protective equipment, including gloves, a laboratory coat, and protective eyewear [1].
 - 2.1.1. WIDE: Talent putting on gloves, a laboratory coat, and protective eyewear at the laboratory bench.
- 2.2. Dilute 100X (*One-Hundred-Ex*) E3 (*E-Three*) stock solution at a 1 to 100 ratio with ultrapure water to prepare a single-strength medium [1].
 - 2.2.1. Talent measuring adding milliliters of 100× E3 stock solution into a beaker with ultrapure water.
- 2.3. Now select sexually mature male and female adult zebrafish [1]. Using breeding tanks with removable dividers, perform controlled breeding [2]. At 8 PM, place male and female zebrafish into separate sections of the breeding tanks at a ratio of 2 males to 3 females, using dividers to keep them apart [3].
 - 2.3.1. Talent selecting adult male and female zebrafish.
 - 2.3.2. Shot of breeding tanks with removable dividers.
 - 2.3.3. Talent transferring the fishes into the breeding tanks.
- 2.4. The next morning, at 8 AM, remove the dividers to allow mating [1]. Collect the embryos and wash them three times with clean system water [2].
 - 2.4.1. Talent removing the dividers from the breeding tanks.
 - 2.4.2. Talent gently washing embryos with system water.
- 2.5. House the embryos in 100-millimeter Petri dishes containing 20 milliliters of fresh E3 medium [1]. Then incubate the embryos at 28 degrees Celsius under a 14-hour light and 10-hour dark cycle [2-TXT].
 - 2.5.1. Talent transferring embryos into 100-millimeter Petri dishes with E3 medium.
 - 2.5.2. Talent placing Petri dishes into an incubator set to 28 degrees Celsius. **TXT: Do not place >50 embryos per dish**

Sleep–Wake Behavioral Analysis of Zebrafish Larvae Using Zebrabox System

NOTE: Please insert the new chapter title here

- 2.6. At 1-day post-fertilization, carefully remove unfertilized, malformed, and abnormal embryos using a Pasteur pipette [1]. Aspirate the medium slowly from the edge of the Petri dish [2] and add fresh E3 embryo medium along the wall of the Petri dish to minimize disturbance to the wildtype embryos [3-TXT].
 - 2.6.1. Talent removing abnormal embryos with a Pasteur pipette.
 - 2.6.2. Talent aspirating medium from the edge of the Petri dish.
 - 2.6.3. Talent adding fresh E3 medium along the wall of the Petri dish. **TXT: For the MPTP-treated group, use 50 μ M MPTP solution; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine**
- 2.7. At 4 days post fertilization, select zebrafish larvae from each group at 6 PM [1]. Then, modify Pasteur pipettes by cutting 10 millimeters from the tip to create a wide bore [2]. Use the modified 3-milliliter Pasteur pipette to transfer one larva per well into a 96-well plate [3].
 - 2.7.1. Talent selecting 4-day post fertilization larvae.
 - 2.7.2. Talent cutting the tip of a Pasteur pipette.
 - 2.7.3. Talent transferring larvae into a 96-well plate using a modified Pasteur pipette.
- 2.8. Observe each well under a stereo microscope to confirm that each zebrafish larva appears healthy [1]. Verify that the fins are open and regularly swinging, the body is upright, and the swim bladder is inflated [2].
 - 2.8.1. LAB MEDIA: 70041_scope-shot_1-.avi: 00:03-00:07
 - 2.8.2. LAB MEDIA: 70041_scope-shot_1-.avi: 00:19-00:27
- 2.9. Next, clean the bottom of the plate carefully to prevent dirt from affecting video tracking results [1]. Then transfer the 96-well plate centrally to the bottom of the Zebrabox chamber [2].
 - 2.9.1. Talent wiping and cleaning the bottom of the 96-well plate.
 - 2.9.2. Talent positioning the 96-well plate inside the Zebrabox chamber.
- 2.10. Select the **Quantization mode** in the video tracking software [1].
 - 2.10.1. LAB MEDIA: 70041_screenshot_1.mp4: 00:05-00:07
- 2.11. For sleep-wake regulation monitoring, set the light adjustment to turn off the light at 10 PM and turn on at 8 AM [1]. Configure the software parameters so that the **detection threshold** is set to **40**, **burst** to **25**, **freeze** to **4**, **bin size** to 60 seconds, and **total monitoring time** to 52 hours [2]. Start the sleep-wake procedure at 8 PM [3].
 - 2.11.1. LAB MEDIA: 70041_screenshot_2.mp4: 00:01-00:16
 - 2.11.2. LAB MEDIA: 70041_screenshot_2.mp4: 00:17-00:36
 - 2.11.3. LAB MEDIA: 70041_screenshot_2.mp4: 00:56-01:00

Assaying Photomotor Response and Thigmotaxis in Zebrafish Larvae

NOTE: Please insert new chapter title here

2.12. To monitor photomotor responses, place 5-day post-fertilization larvae into a 96-well plate with one larva per well [1]. Then set the light transition program to 0 to 60 seconds of darkness followed by 60 to 120 seconds of constant light [2].

2.12.1. Talent placing individual larvae into each well of a 96-well plate.

2.12.2. LAB MEDIA: 70041_screenshot_3.mp4: 00:01-00:03, 00:08-00:10, 00:17-00:35

2.13. Start the protocol and collect motion parameters every 6 seconds throughout the 120-second assay [1]. Stop the protocol at 120 seconds and export the data file [2].

2.13.1. LAB MEDIA: 70041_screenshot_4.mp4: 00:02-00:10

2.13.2. LAB MEDIA: 70041_screenshot_5.mp4: 00:06-00:16

2.14. Then place the larvae in complete darkness for 5 minutes to acclimate them for the subsequent dark strobe light assay [1]. Set the light transition program to 0 to 60 seconds of darkness followed by 60 to 120 seconds of strobe light at 10 hertz [2].

2.14.1. Talent placing larvae in full darkness.

2.14.2. LAB MEDIA: 70041_screenshot_6.mp4: 00:14-00:26

2.15. To assess thigmotaxis, select two 6-well plates [1]. Fill each well with 4 milliliters of E3 medium [2]. At 5 days post-fertilization, collect zebrafish larvae from the wild type group and the MPTP-treated group [3] and place them into separate 6-well plates [4].

2.15.1. Shot of 2 6-well plates.

2.15.2. Talent filling 6-well plates with E3 medium.

2.15.3. Talent collecting larvae from wild type and test groups. ISin

2.15.4. Talent transferring larvae into separate 6-well plates.

2.16. Before the thigmotaxis assessment, place the well plates in the Zebrabox for 15 minutes to allow acclimation [1]. Then use the **Draw Area** function to draw two concentric circles in each well, with the inner circle radius equal to half the radius of the well, and start assessment [2].

2.16.1. Talent placing plates into the Zebrabox chamber.

2.16.2. LAB MEDIA: 70041_screenshot_7.mp4: 00:08-00:12, 00:45-00:51, 01:22-01:29

Evaluation of Locomotor Activity, Startle Response, and Light–Dark Responses in Zebrafish

NOTE: Please insert new chapter title here

2.17. For locomotor assessment, transfer 5-day-old zebrafish larvae from the wild type group and the MPTP (*M-P-T-P*)-treated group into two separate 12-well plates [1]. After a 15-minute acclimation, select the **Tracking-online analysis** mode [2] and record locomotor activity for 5 minutes using the automated video-tracking system [3].

2.17.1. Talent transferring larvae into 12-well plates.

2.17.2. LAB MEDIA: 70041_screenshot_8.mp4: 00:05-00:10

2.17.3. LAB MEDIA: 70041_screenshot_9.mp4: 00:02-00:20

2.18. For the startle response test, transfer 5-day-old larvae individually into two 12-well plates with one larva per well and 2 milliliters of E3 medium [1]. Place the plates into the Zebrabox for a 5-minute light acclimation period [2].

2.18.1. Talent transferring larvae into 12-well plates.

2.18.2. Talent placing plates into the Zebrabox chamber.

2.19. Launch the Zebralab software and activate the **Tracking-Online Analysis** mode [1]. Open the **Light-triggering** interface, then select **Use one of the 3 triggering methods below** and enable **Enhanced stimuli** [2]. Now set to **When session is started** [3].

2.19.1. LAB MEDIA: 70041_screenshot_10.mp4: 00:04-00:07, 00:19-00:22

2.19.2. LAB MEDIA: 70041_screenshot_10.mp4: 00:32-00:35

2.19.3. LAB MEDIA: 70041_screenshot_10.mp4: 00:35-00:38

2.20. In the transition schedule, define the dark flash rhythm by setting the **Transition 1** to **5 ms (milliseconds)** with **100%** light intensity; **transition 2** to **2890 ms** with 100% light intensity, **transition 3** to **5 ms** with 0% light intensity and **transition 4** to **100 ms** with 0% light intensity [1]. Tick the box for **Repeat list of transitions** to ensure periodic flashing and then start the protocol [2].

2.20.1. LAB MEDIA: 70041_screenshot_11.mp4: 00:01-00:08 *Video editor: please pause the video and highlight the parameters on the left side of the video*

2.20.2. LAB MEDIA: 70041_screenshot_11.mp4: 00:18-00:25, 00:48-00:52

2.21. For the light-dark challenge assay, transfer zebrafish larvae into two 24-well plates with 1 larva per well and 1 milliliter of E3 medium [1]. After a 5-minute acclimation in the Zebrabox, turn on the **Tracking Analysis Online** mode, then configure the light program for 6 minutes of constant light followed by 4 minutes of complete darkness [2-TXT].

2.21.1. Shot of zebrafish larva in 2 24-well plates with E3 medium.

2.21.2. LAB MEDIA: 70041_screenshot_12.mp4: 00:02-00:05, 00:35-01:01 *Video editor: Please speed up the video*

2.22. At the tenth minute, stop behavioral recording and save the data [1]. Carefully remove the plate from the Zebrabox [2].

2.22.1. LAB MEDIA: 70041_screenshot_13.mp4: 00:02-00:11

2.22.2. Talent removing the plate from the Zebrabox.

2.23. After each behavioral experiment, spray and wipe all MPTP-contacted surfaces with freshly prepared sodium hypochlorite solution [1].

2.23.1. Talent disinfecting lab surfaces and the Zebrabox with bleach solution.

2.24. Collect liquid and solid waste containing MPTP and clearly label them as hazardous neurotoxic waste [1]. Treat all contaminated materials with freshly prepared sodium hypochlorite solution at [2-TXT].

2.24.1. Talent labeling MPTP waste containers.

2.24.2. Talent treating waste with bleach solution. **TXT: Euthanize MPTP-treated zebrafish in diluted sodium hypochlorite solution**

Results

3. Results

- 3.1. Treated zebrafish larvae showed disrupted sleep and wake activity traces compared to the wild-type group [1-TXT]. Total sleep time was also significantly increased in the treated group [2].
 - 3.1.1. LAB MEDIA: Figure 2.
Video editor: Highlight the MPTP traces (red) in all graphs
 - 3.1.2. LAB MEDIA: Figure 3C. *Video editor: Please highlight the bars corresponding to MPTP (grey).*
- 3.2. The number of sleep bouts was significantly decreased in the treated group [1], while the average sleep bout length increased [2].
 - 3.2.1. LAB MEDIA: Figure 3E. *Video editor: Please highlight the bars corresponding to MPTP (grey).*
 - 3.2.2. LAB MEDIA: Figure 3D. *Video editor: Please highlight the bars corresponding to MPTP (grey).*
- 3.3. Wake activity and average activity were significantly impaired in the treated group [1].
 - 3.3.1. LAB MEDIA: Figure 3A and B *Video editor: Please highlight the bars corresponding to MPTP (grey).*
- 3.4. Under constant light, the freezing index of the treated group was significantly increased [1]. It also significantly increased under strobe light stimulation at 10 hertz [2].
 - 3.4.1. LAB MEDIA: Figure 4 A and B. *Video editor: Please highlight the bars corresponding to MPTP (red) in B and the red curve in A*
 - 3.4.2. LAB MEDIA: Figure 4C and D. *Video editor: Please highlight the bars corresponding to MPTP (red) in D and the red curve in C*
- 3.5. Photomotor response curves showed reduced reactivity in the treated group [1].
 - 3.5.1. LAB MEDIA: Figure 4A,C. *Video editor: Please highlight the red curves*
- 3.6. Tracking results indicated reduced locomotor and exploratory behavior in the treated group [1]. Total swimming [2] and average swimming speed were also significantly reduced in the treated group [3].
 - 3.6.1. LAB MEDIA: Figure 6A. *Video editor: Please highlight the rows of plates corresponding to MPTP*
 - 3.6.2. LAB MEDIA: Figure 6C. *Video editor: Please highlight the bars corresponding to MPTP (grey)*

- 3.6.3. LAB MEDIA: Figure 6D. *Video editor: Please highlight the bars corresponding to MPTP (grey)*
- 3.7. The freeze movement time ratio was significantly higher in the treated group [1], but the rapid movement time ratio was significantly lower [2].
- 3.7.1. LAB MEDIA: Figure 6E. *Video editor: Please highlight the bars corresponding to MPTP (grey)*
- 3.7.2. LAB MEDIA: Figure 6F. *Video editor: Please highlight the bars corresponding to MPTP (grey)*
- 3.8. Alternating light and dark transitions produced pronounced velocity peaks in wild-type larvae [1]. The distance swum after stimulation [2] and peak velocity after stimulation were significantly decreased in the treated group [3].
- 3.8.1. LAB MEDIA: Figure 7A. *Video editor: Please highlight the curve corresponding to WT (blue).*
- 3.8.2. LAB MEDIA: Figure 7B. *Video editor: Please highlight the bars corresponding to MPTP (grey)*
- 3.8.3. LAB MEDIA: Figure 7C. *Video editor: Please highlight the bars corresponding to MPTP (grey)*
- 3.9. Latency to peak speed movement onset was also significantly delayed in the treated group [1].
- 3.9.1. LAB MEDIA: Figure 7D. *Video editor: Please highlight the bars corresponding to MPTP (grey).*

1. Zebrafish
Pronunciation link: <https://www.howtopronounce.com/zebra-fish>
IPA: /z' i:.bɪ.ə fɪʃ/
Phonetic Spelling: zee·bruh·fish
2. Embryo
Pronunciation link: <https://www.merriam-webster.com/dictionary/embryo>
IPA: /' embri oo/
Phonetic Spelling: em·bree·oh
3. Petri dish
Pronunciation link: <https://www.merriam-webster.com/dictionary/petri%20dish>
IPA: /' pi:tri dɪʃ/
Phonetic Spelling: pee·tree·dish

4. Incubate
Pronunciation link: <https://www.merriam-webster.com/dictionary/incubate>
IPA: /'ɪŋkjə,bert/
Phonetic Spelling: ing·kyuh·bayt
5. Fertilization
Pronunciation link: <https://www.merriam-webster.com/dictionary/fertilization>
IPA: /,fɜːtə,lə'zeɪʃən/
Phonetic Spelling: fur·tuh·luh·zay·shuhn
6. Larvae
Pronunciation link: <https://www.merriam-webster.com/dictionary/larva>
IPA: /'larvi:/, /'larvai/
Phonetic Spelling: lar·vee, lar·vye
7. Acclimation
Pronunciation link: <https://www.merriam-webster.com/dictionary/acclimation>
IPA: /,æklə'meɪʃən/, /,æklai'meɪʃən/
Phonetic Spelling: ak·luh·may·shuhn, ak·lye·may·shuhn
8. Thigmotaxis
Pronunciation link: <https://www.howtopronounce.com/thigmotaxis>
IPA: /θɪ.gm.ə.t'æksɪs/
Phonetic Spelling: thig·muh·tak·sis
9. Locomotor
Pronunciation link: <https://www.merriam-webster.com/dictionary/locomotor>
IPA: /,ləʊkə'moʊtər/
Phonetic Spelling: loh·kuh·moh·tur
10. Hypochlorite
Pronunciation link: <https://www.merriam-webster.com/dictionary/hypochlorite>
IPA: /,haɪpə'klɔʊə,aɪt/, /,haɪpə'klɔrə,aɪt/
Phonetic Spelling: hye·puh·klor·uh·eyet, hye·puh·klawr·uh·eyet
11. Euthanize
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /'ju:θə,naɪz/
Phonetic Spelling: yoo·thuh·nyz
12. Malformed
Pronunciation link: <https://www.merriam-webster.com/dictionary/malformed>
IPA: /,mæl'fɔrmd/
Phonetic Spelling: mal·formd