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Scriptwriter Name: Sri Lakshmi Priya

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Title: Microinjection of Interleukin-6 into the Bloodstream of TSC Zebrafish Larvae to Study Neuropsychiatric Disorder-Like Behaviors

Authors and Affiliations:

Shivani Dogra^{1,2}, Justyna Zmorzynska¹

¹Laboratory of Developmental Neurobiology, International Institute of Molecular Mechanisms and Machines, IMol, Polish Academy of Sciences

²Warsaw-4-PhD doctoral school, International Institute of Molecular and Cell Biology in Warsaw

Corresponding Authors:

Justyna Zmorzynska j.zmorzynska@imol.institute

Email Addresses for All Authors:

Shivani Dogra s.dogra@imol.institute

Justyna Zmorzynska j.zmorzynska@imol.institute

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**

If **Yes**, can you record movies/images using your own microscope camera?

No

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

Ok, we have a very old Leica stereo microscope with a broken camera, but the port is working. We are planning to replace the camera, but the procurement takes time.

LEICA M165 FC

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

Scope shots: 2.6.3, 2.7.2, 2.8.2, 3.1.1, 3.3.1, 3.3.2, 3.5.1, 3.5.2, 3.5.3, 3.7.1

Videographer: Please film above mentioned shots using a scope kit.

NOTE: The scope shots were filmed by the videographer using a scope kit.

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

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. **Yes**

If **Yes**, please provide the **full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author willing to participate.

Shivani Dogra, PhD Student

Current Protocol Length

Number of Steps: 16

Number of Shots: 36

Interviews

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

INTRODUCTION:

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

- 1.1. **Shivani:** My research investigates how IL-6-induced inflammation interacts with genetic risk mutations for autism to influence brain development and function.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Shivani:** Unlike broad LPS-induced inflammation, this protocol selectively elevates IL-6, enabling precise investigation of cytokine-specific neurodevelopmental effects.
 - 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. **Shivani:** This protocol enables controlled IL-6 inflammation studies to assess gene-environment effects on neurodevelopment and behavior in zebrafish.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

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

- 1.4. **Shivani:** Key considerations when performing this protocol include consistent needle size, precise 2nL injection volume, and minimizing larval time outside E3.
 - 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. **Shivani:** Future studies can investigate how IL-6 inflammation and genetic risk together affect brain development and behavior.

- 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.

~~What value do you expect this video/article to have for other researchers working in your field?~~

- 1.6. **Shivani Dogra, PhD Student**: (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.

Videographer's Notes: Shots 1.6.1 and 1.7.1 recorded twice - once according to the instructions, the second time the talent speaks directly to the camera

~~How does the video format of your article help you to share your work with the broader scientific community?~~

- 1.7. **Shivani Dogra, PhD Student**: (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.

Protocol

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

2. Preparation of Agarose Plates and Dechorionated Larvae

Demonstrator: Shivani Dogra

- 2.1. To begin, gather the microinjection needles, required reagents, and 2-day post-fertilization or dpf (*D-P-F*) zebrafish embryos [1].
 - 2.1.1. Talent walking into the room with a tray of required materials. ***Videographer:** Please obtain CU shot as well for this shot.* **Videographer's Notes:** An extra shot added as a close-up to be placed right after 2.1.1
- 2.2. Prepare a 1% weight per volume agarose solution in E3 (*E-three*) medium [1] and heat it until the agarose is completely dissolved and the solution becomes clear [2].
 - 2.2.1. Talent adding agarose into the bottle containing E3 medium.
 - 2.2.2. Talent heating the agarose solution until the solution becomes clear.
- 2.3. Allow the agarose solution to cool briefly [1]. Pour approximately 30 milliliters into a 100-millimeter Petri dish to ensure an even layer across the surface [2].
 - 2.3.1. Talent placing the agarose solution on workbench to cool.
 - 2.3.2. Talent pouring the agarose solution into a 100mm Petri dish to form an even layer.
- 2.4. While the agarose is still liquid but beginning to solidify, gently place a silicone mold onto the surface to create uniform grooves [1].
 - 2.4.1. Talent placing a silicone mold onto the partially solidified agarose surface.
- 2.5. Transfer approximately 100 chorionated embryos at 2 dpf into a small 50-millimeter agarose-coated Petri dish [1].
 - 2.5.1. Talent transferring chorionated embryos into a 50 mm agarose-coated Petri dish.
- 2.6. Add 4 milliliters of freshly prepared pronase solution to the dish, ensuring the embryos are fully submerged [1]. Incubate at room temperature for 3 to 5 minutes [2]. Monitor the release of embryos from the chorions under a stereomicroscope [3].
 - 2.6.1. Talent adding pronase solution to the dish till the embryos are submerged.
 - 2.6.2. Talent placing the dish on the benchtop for incubation.
 - 2.6.3. SCOPE: Shot of embryos being released from the chorions

- 2.7. Gently rotate or swirl the dish intermittently to facilitate enzymatic removal of the chorions [1]. Continue until the majority of embryos are released from their chorions [2].

2.7.1. SCOPE: ~~Talent gently swirling~~ Show the Petri dish being swirled. Author's NOTE: Talent shot was changed to SCOPE shot, and 2.6.3 was combined with 2.7.1. Videographer's NOTE: 2.6.1 and 2.7.1- recorded in one file, one take

NOTE: The videographer's Note and the author's note don't match. Since both are scope shots, go with the author's notes.

2.7.2. SCOPE: Show the embryos being released from their chorions.

- 2.8. Swirl the Petri dish to make sure all the embryos are out of the chorions [1]. Wash the embryos thoroughly by transferring them through 5 to 6 changes of fresh E3 embryo medium [2]. Ensure that all chorions and pronase residues are removed to prevent damage to the larvae and to maintain optimal culture conditions [3]. NOTE: VO is added for the added shot, and the sentence numbers are adjusted to accommodate the added shot.

Added shot 2.8.0: Talent swirling the Petri dish, showing that the embryos are out of the chorions.

2.8.1. Talent transferring embryos through multiple dishes containing fresh E3 embryo medium.

2.8.2. SCOPE: Show dechorionated embryos free of chorions and pronase residues.

- 2.9. Using a glass Pasteur pipette, transfer approximately 50 dechorionated larvae into a 100-millimeter Petri dish containing 40 milliliters of E3 medium [1]. Maintain the larvae in E3 medium until microinjection [2].

2.9.1. Talent transferring dechorionated larvae into a 100-millimeter Petri dish containing E3 medium using a glass Pasteur pipette.

2.9.2. Shot of larvae maintained in E3 embryo medium.

3. Microinjection of Zebrafish Larvae

- 3.1. Under a stereomicroscope, use fine forceps to carefully break the tip of the previously prepared glass capillary to generate a micropipette with an opening of approximately 3 micrometers in diameter [1]. Prepare an injection solution of a 0.9% weight per volume sodium chloride control solution, and use the previously prepared interleukin-6 or IL-6 (*l-l-six*) solution at 2 picograms per nanoliter in 0.9% sodium chloride [2]. Add 1 microliter of 0.05% trypan blue to every 10 microliters of solution to facilitate visualization during injection [3].

3.1.1. SCOPE: Shot of breaking the tip of the glass capillary using fine forceps.

- 3.1.2. Talent preparing the injection solutions.
- 3.1.3. Talent adding trypan blue to the injection solution.
- 3.2. With a pipette, load the solution into the microladder tip [1]. Using a microloader tip, backfill the prepared micropipette injection needle with approximately 3 microliters of injection solution, ensuring no air bubbles are introduced [2]. Secure the loaded micropipette injection needle into the capillary holder connected to the microinjection system [3]. Place a small drop of mineral oil onto a calibration slide [4]. NOTE: VO is added for the added shot, and the sentence numbers are adjusted to accommodate the added shot.

Added shot 3.2.0: Talent loading solution into microladder tip using pipette

- 3.2.1. Talent loading the injection solution into the micropipette injection needle using a microloader tip.
- 3.2.2. Talent securing the loaded micropipette injection needle into the capillary holder.
- 3.2.3. Talent placing a drop of mineral oil onto a calibration slide.
- 3.3. Calibrate the injection volume by expelling droplets into the mineral oil [1]. Measure the droplet diameter using the calibration slide with a 0.01-millimeter scale [2]. Adjust the injection pressure to achieve a droplet volume of approximately 1 nanoliter [3].
 - 3.3.1. SCOPE: Talent expelling Show the droplets being expelled into mineral oil. Videographer's Notes: 3.3.1 and 3.3.2 recorded in one file/take. Author's NOTE: Talent shot was changed to scope shot.
 - 3.3.2. SCOPE: Show measurement of droplet diameter on the calibration slide with a 0.01mm scale.
 - 3.3.3. Close-up shot of the injection pressure settings being adjusted in the microinjector screen.
- 3.4. Anesthetize the larvae in small batches by adding tricaine to the E3 medium at a final 1x concentration to minimize stress and ensure consistent sedation [1]. Transfer individual 2 dpf larvae onto a solidified agarose injection plate [2]. Place the dish under the stereomicroscope [3]. Using a glass pipette, gently orient each larva so that the duct of Cuvier is clearly visible and accessible [4].
 - 3.4.1. Talent adding tricaine to the E3 embryo medium containing larvae.
 - 3.4.2. Talent transferring larvae onto the agarose injection plate.
 - 3.4.3. Talent placing the dish under the stereomicroscope.
 - 3.4.4. Talent orienting a larva with a glass pipette.
- 3.5. Position the micropipette tip at the entry point of the duct of Cuvier by tracking blood cells moving toward the heart under the stereomicroscope [1]. Inject the first droplet

of the prepared solution into the duct of Cuvier [2]. After approximately 20 seconds, inject a second droplet into the duct of Cuvier [3].

3.5.1. SCOPE: Show the micropipette positioned at the duct of Cuvier while blood cells are visible moving toward the heart.

3.5.2. SCOPE: Show the first injection into the duct of Cuvier. Videographer's Notes: 3.5.2 and 3.5.3 recorded in one file/take

3.5.3. SCOPE: Show the second injection into the duct of Cuvier.

3.6. Following injection, gently transfer the larvae into a fresh 100-millimeter Petri dish containing 40 milliliters of E3 medium [1]. Return the larvae to an incubator at 28.5 degrees Celsius for recovery [2].

3.6.1. Talent transferring injected larvae into a fresh 100mm Petri dish containing E3 medium.

3.6.2. Talent placing the Petri dish into the incubator.

3.7. On the following day, repeat the microinjection procedure as previously demonstrated [1]. At 5 dpf, use the larvae for downstream applications, including behavioral assays, brain imaging, or immunofluorescence analysis [2].

3.7.1. SCOPE: Shot of 3 dpf larvae being prepared for the second microinjection.

3.7.2. Shot of larvae ready for downstream analyses.

Results

4. Results

- 4.1. IL-6 microinjection did not affect larval viability [1] compared to sodium chloride injections [2].
 - 4.1.1. LAB MEDIA: Figure 4A. *Video editor: Emphasize the grey line with its datapoints labeled il6*
 - 4.1.2. LAB MEDIA: Figure 4A. *Video editor: Emphasize the blue line with its datapoints labeled NaCl*
- 4.2. Survival rates at 5 dpf were indistinguishable between IL-6–injected [1] and NaCl (N-A-C-L)-injected groups [2], indicating that the dosing regimen is well tolerated and does not introduce nonspecific toxicity [3].
 - 4.2.1. LAB MEDIA: Figure 4A. *Video editor: Emphasize the Day 5 endpoint of the grey line labeled il6*
 - 4.2.2. LAB MEDIA: Figure 4A. *Video editor: Emphasize the Day 5 endpoint of the blue line labeled NaCl*
 - 4.2.3. LAB MEDIA: Figure 4A.
- 4.3. Microinjections slightly reduced viability [1] compared to uninjected siblings [2].
 - 4.3.1. LAB MEDIA: Figure 4A. *Video editor: Emphasize the Day 5 endpoint of the grey line labeled il6 and blue line labeled NaCl*
 - 4.3.2. LAB MEDIA: Figure 4A. *Video editor: Emphasize the Day 5 endpoint of the dark blue line labeled Control*
- 4.4. The homozygous tuberous sclerosis complex 2 or tsc2 (T-S-C-two) mutants displayed significantly higher baseline prostaglandin E2 or PGE2 (P-G-E-two) levels [1] than wild-type siblings [2].
 - 4.4.1. LAB MEDIA: Figure 4B. *Video editor: Emphasize the magenta tsc2-/- boxplot in NaCl.*
 - 4.4.2. LAB MEDIA: Figure 4B. *Video editor: Emphasize the blue tsc2+/+ boxplot in NaCl.*
- 4.5. Heterozygous tsc2 mutants showed a modest, non-significant increase in baseline PGE2 [1] compared with wild-type siblings [2].
 - 4.5.1. LAB MEDIA: Figure 4B. *Video editor: Emphasize the green tsc2+/- boxplot in NaCl.*
 - 4.5.2. LAB MEDIA: Figure 4B. *Video editor: Emphasize the blue tsc2+/+ boxplot in NaCl.*
- 4.6. PGE2 levels rose sharply in heterozygous tsc2 mutants following IL-6 microinjection [1].

- 4.6.1. LAB MEDIA: Figure 4B. *Video editor: Emphasize the green tsc2+/- boxplot in IL6.*
- 4.7. The open field test showed that tsc2 mutants displayed increased anxiety-like behavior [1] compared with wild-type siblings [2]. However, IL6 microinjections did not affect this behavior [3].
 - 4.7.1. LAB MEDIA: Figure 4C. *Video editor: In NaCl panel, emphasize the green tsc2+/- and magenta tsc2-/- boxplots*
 - 4.7.2. LAB MEDIA: Figure 4C. *Video editor: In NaCl panel, emphasize the blue tsc2+/+ boxplots*
 - 4.7.3. LAB MEDIA: Figure 4C. *Video editor: Emphasize the IL6 panel.*
- 4.8. The heterozygous tsc2 and homozygous tsc2 mutants spent significantly more time in the outer zone of the arena and exhibited reduced center exploration [1] than wild-type siblings [2].
 - 4.8.1. LAB MEDIA: Figure 4C. *Video editor: In NaCl panel, emphasize the green tsc2+/- and magenta tsc2-/- boxplots*
 - 4.8.2. LAB MEDIA: Figure 4C. *Video editor: In NaCl panel, emphasize the blue tsc2+/+ boxplots.*
- 4.9. IL-6 injections increased repetitive, stereotyped swimming patterns, particularly in heterozygous tsc2 mutants [1].
 - 4.9.1. LAB MEDIA: Figure 4D
- 4.10. The proportion of IL-6-injected heterozygous tsc2 mutants exhibiting repetitive looping was significantly increased [1] compared with sodium chloride-injected controls [2].
 - 4.10.1. LAB MEDIA: Figure 4E. *Video editor: In repetitive panel, tsc2+/- row, emphasize the green IL6 boxplot showing approximately 10 fish.*
 - 4.10.2. LAB MEDIA: Figure 4E. *Video editor: In repetitive panel, tsc2+/- row, emphasize the green NaCl boxplot showing approximately 2 to 3 fish.*

Pronunciation guides:

1. Microinjection
 Pronunciation link: <https://www.merriam-webster.com/dictionary/microinjection>
 IPA: /ˌmaɪkroʊɪnˈdʒɛkʃən/
 Phonetic Spelling: MY·kroh·in·JEK·shuhn
2. Fertilization
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fertilization>
 IPA: /ˌfɜːtəlaɪˈzeɪʃən/
 Phonetic Spelling: FUR·tuh·luh·ZAY·shuhn

3. Zebrafish
Pronunciation link: <https://www.merriam-webster.com/dictionary/zebrafish>
IPA: /'zi:brəˌfɪʃ/
Phonetic Spelling: ZEE·bruh·fish
4. Agarose
Pronunciation link: <https://www.merriam-webster.com/dictionary/agarose>
IPA: /'ægəˌroʊs/
Phonetic Spelling: AG·uh·rohs
5. Silicone
Pronunciation link: <https://www.merriam-webster.com/dictionary/silicone>
IPA: /'sɪlɪˌkoʊn/
Phonetic Spelling: SIL·ih·kohn
6. Chorionated
Pronunciation link: No confirmed link found
IPA: /'kɔ:riəˌneɪtɪd/
Phonetic Spelling: KOR·ee·uh·nay·tid
7. Pronase
Pronunciation link: <https://www.howtopronounce.com/pronase>
IPA: /'proʊneɪs/
Phonetic Spelling: PROH·nays
8. Stereomicroscope
Pronunciation link: <https://www.howtopronounce.com/stereomicroscope>
IPA: /ˌstɛrɪəʊˈmaɪkrəˌskoʊp/
Phonetic Spelling: STAIR·ee·oh·MY·kruh·skohp
9. Enzymatic
Pronunciation link: <https://www.merriam-webster.com/dictionary/enzymatic>
IPA: /ˌɛnzəˈmætɪk/
Phonetic Spelling: EN·zy·MAT·ik
10. Dechorionated
Pronunciation link: No confirmed link found
IPA: /diːˈkɔ:riəˌneɪtɪd/
Phonetic Spelling: dee·KOR·ee·uh·nay·tid
11. Capillary
Pronunciation link: <https://www.merriam-webster.com/dictionary/capillary>
IPA: /'kæpəˌlɛri/
Phonetic Spelling: KAP·uh·lair·ee
12. Micropipette
Pronunciation link: <https://www.merriam-webster.com/dictionary/micropipette>
IPA: /ˌmaɪkrəʊˈpaɪˌpɛt/
Phonetic Spelling: MY·kroh·py·PET
13. Interleukin-6
Pronunciation link: <https://www.merriam-webster.com/dictionary/interleukin>

IPA: /ˌɪntərˈluːkɪn/

Phonetic Spelling: IN·ter·LOO·kin six

14. Picogram

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

IPA: /ˈpiːkoʊˌɡræm/

Phonetic Spelling: PEE·koh·gram

15. Trypan

Pronunciation link: <https://www.howtopronounce.com/trypan>

IPA: /ˈtraɪpæn/

Phonetic Spelling: TRY·pan

16. Microloader

Pronunciation link: No confirmed link found

IPA: /ˌmaɪkroʊˈloʊdər/

Phonetic Spelling: MY·kroh·LOH·der

17. Backfill

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

IPA: /ˈbæk fɪl/

Phonetic Spelling: BACK·fill

18. Microliter

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

IPA: /ˈmaɪkroʊˌliːtər/

Phonetic Spelling: MY·kroh·LEE·ter

19. Calibration

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

IPA: /ˌkæləˈbreɪʃən/

Phonetic Spelling: KAL·uh·BRAY·shuhn

20. Anesthetize

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

IPA: /əˈnɛsθəˌtaɪz/

Phonetic Spelling: uh·NES·thuh·tyze

21. Tricaine

Pronunciation link: <https://www.howtopronounce.com/tricaine>

IPA: /traɪˈkeɪn/

Phonetic Spelling: try·KAYN

22. Duct of Cuvier

Pronunciation link: <https://www.howtopronounce.com/cuvier>

IPA: /ˈkjuːviər/

Phonetic Spelling: kyoo·vee·AY

23. Behavioral

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

IPA: /bɪˈheɪvjərəl/

Phonetic Spelling: bih·HAYV·yer·uhl

24. Immunofluorescence
Pronunciation link: <https://www.merriam-webster.com/dictionary/immunofluorescence>
IPA: /ɪˌmjuːnoʊfloʊˈresəns/
Phonetic Spelling: ih·MYOO·noh·floor·ESS·uhns
25. Homozygous
Pronunciation link: <https://www.merriam-webster.com/dictionary/homozygous>
IPA: /ˌhoʊmoʊˈzaɪɡəs/
Phonetic Spelling: HOH·moh·ZY·guhs
26. Tuberous
Pronunciation link: <https://www.merriam-webster.com/dictionary/tuberous>
IPA: /ˈtuːbəɪs/
Phonetic Spelling: TOO·buh·ruhs
27. Sclerosis
Pronunciation link: <https://www.merriam-webster.com/dictionary/sclerosis>
IPA: /skləˈroʊsɪs/
Phonetic Spelling: skluh·ROH·suhs
28. Prostaglandin
Pronunciation link: <https://www.merriam-webster.com/dictionary/prostaglandin>
IPA: /ˌprɑːstəˈglændən/
Phonetic Spelling: PROS·tuh·GLAN·din
29. Heterozygous
Pronunciation link: <https://www.merriam-webster.com/dictionary/heterozygous>
IPA: /ˌhetərəˈzaɪɡəs/
Phonetic Spelling: HET·uh·roh·ZY·guhs
30. Baseline
Pronunciation link: <https://www.merriam-webster.com/dictionary/baseline>
IPA: /ˈbeɪsˌlaɪn/
Phonetic Spelling: BAYS·line
31. Anxiety
Pronunciation link: <https://www.merriam-webster.com/dictionary/anxiety>
IPA: /æŋˈzaɪəti/
Phonetic Spelling: ang·ZY·uh·tee
32. Stereotyped
Pronunciation link: <https://www.merriam-webster.com/dictionary/stereotyped>
IPA: /ˈstəriəˌtaɪpt/
Phonetic Spelling: STAIR·ee·oh·typed
33. Looping
Pronunciation link: <https://www.merriam-webster.com/dictionary/looping>
IPA: /ˈluːpɪŋ/
Phonetic Spelling: LOO·ping