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Title: In Vivo Imaging of Fully Active Brain Tissue in Awake Zebrafish Larvae and Juveniles by Skull and Skin Removal

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **Y**

If you require a microscope but can record movies/images through your microscope with your own camera, please indicate **Yes** here: **Y**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

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? **Please select one**.

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

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **10**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Paul Schramm**: This method allows the visualization of zebrafish brain tissue in later larval stages to allow observation of the neuronal architecture in a highly detailed fashion in vivo that has previously not been possible **[1]**.

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

REQUIRED:

1.2. **Paul Schramm**: Pigments cells, which emerge during later stages of larval development and prevent clear imaging of the brain, are not a problem with this method because they are simply removed **[1]**.

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

OPTIONAL:

1.3. **Reinhard W. Köster**: This method can be used to shed light on the biological mechanisms of neurodegeneration and regeneration, processes that occur in mature neurons and that impact synaptic plasticity **[1]**.

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

Protocol

2. Larva Preparation

2.1. Before beginning the experiment, use a micropipette puller to prepare sharp, thin glass needles **[1-TXT]** from glass capillaries **[added 2]**.

2.1.1. WIDE: Talent pulling needle **TEXT: See text for micropipette puller setting details**

2.1.2. Added shot: CU: Talent pulling needle

2.2. Next, use a plastic Pasteur pipette to collect larvae into a 90-millimeter-diameter Petri dish containing the appropriate solution **[1-TXT]**.

2.2.1. Talent adding larvae to dish **TEXT: i.e., Danieau for larvae maintained in Danieau; fish facility water for >7 dpf larvae**

2.3. Transfer the selected larvae to a 35-millimeter-diameter Petri dish containing ACSF *(A-C-S-F)* **[1-TXT]** and place a 24- x 24-millimeter, square, glass coverslip into the Petri dish lid **[2]**.

2.3.1. Talent adding larvae to dish, with ACSF container visible in frame **TEXT: ACSF: artificial cerebrospinal fluid**

2.4. Then aliquot the volume of ACSF needed for the experiment into an appropriate vial for oxygenation with carbogen **[1-TXT]**.

2.4.1. Talent adding medium to vial and/or oxygenating medium **TEXT: See text for all medium and solution preparation details**

3. Larva Embedding

3.1. To embed the larva, use a Pasteur pipette to transfer the anesthetized larvae to a mounting chamber **[1-TXT]** under a stereo microscope **[added 2]**. If any larvae are still able to move, do not use the fish for the experiment until the fish are completely unable to move **[3]**.

3.1.1. WIDE: Talent adding larvae to chamber **TEXT: See text for mounting chamber preparation and larva anesthesia details**

3.1.2. Added shot: Close up: Talent adding larvea to chamber

3.1.3. LAB MEDIA: 1: 00:03-00:08

3.2. When the larvae are immobile, carefully remove the excess medium **[1]** and immediately add at least 1 milliliter of low melting agarose onto the larvae **[2]**.

3.2.1. LAB MEDIA: 2.1: 00:40-00:47

3.2.2. Talent adding agarose to larvae

3.3. Orient the zebrafish with the dorsal region facing up as close to the surface of the agarose as possible **[1]**.

3.3.1. LAB MEDIA: 2.2.: 01:58-02:10

3.4. If the larvae will be imaged using an inverted microscope, after solidifying, trim the agarose containing the larvae into a small cuboid block **[1-TXT]**.

3.4.1. LAB MEDIA: 3: 00:07-00:17 **TEXT: Trimming not necessary for upright microscope**

4. Brain Exposure

4.1. To expose the brain for imaging, trim away the excess agarose **[1]** over the brain region of interest as necessary **[added 4.1.1.1]** and use a glass needle to make a small incision through the skin near but not over the region of interest without penetrating too deeply into the tissue **[2]**.

4.1.1. WIDE: Talent trimming agarose. *Videographer: Important step*

4.1.1.1. Added shot: CU: Talent trimming agarose. *Videographer: Important step*

4.1.2. LAB MEDIA: 4.1: 00:09-00:19

4.2. Barely moving the needle just under the skin surface, continue to carefully make very small cuts around the region of interest until the skin over the region of interest can be removed or pushed aside **[1-TXT]**.

4.2.1. LAB MEDIA: 4.1: 00:19-00:35 **TEXT: Use oxygenated ACSF to flush away unwanted skin particles and blood**

4.3. When the tissue has been removed from all of the embryos **[1]**, add a small drop of low melting agarose to the previously prepared imaging chamber of an inverted microscope **[2-TXT]** and use a small spatula to flip the cuboid agarose block 180 degrees onto the agarose drop in the imaging chamber **[3]**.

4.3.1. LAB MEDIA: 5: 00:00-00:02

4.3.2. Talent adding agarose to chamber **TEXT: See text for upright microscope imaging details**

4.3.3. Cuboid being added to chamber

4.4. When the agarose has solidified, fill the imaging chamber with fresh ACSF **[1]** and begin imaging the larvae **[2]**.

4.4.1. Talent filling chamber with ACSF

4.4.2. LAB MEDIA: 6.1: 00:13-00:23

4.4.3. Added shot: WIDE: Talent at microscope, imaging larva, with monitor visible in frame, checking the final data

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see? Please list 4 to 6 individual steps.
4.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success? Please list 1 or 2 individual steps from the script above.
4.2.1. and 4.2.2. are by far the most important/critical steps. If something goes wrong during this micro-neuro-surgery the experimenter will not be able to investigate physiologic "healthy" brain activity. To ensure success it is important to have calm and not shaky hands and also to work extremely precise. It's better to be too careful and rather cut into the agarose/air above the skull then to cut and injure the underlying brain tissue. So, the person performing this protocol should be aware that this step is the most important step and if something goes wrong here the worst-case scenario would be that the fish dies because of lethal brain damage/hemorrhage so always focus 100% when performing step 4.2!
Also, training is necessary (like for every other surgery) to raise the success rate and optimize performance.

Results

5. Results: Representative In Vivo Fully Active Awake Zebrafish Larva and Juvenile Brain Tissue Imaging

5.1. Here an intact 14-days-post-fertilization transgenic larva with the skull still intact can be observed **[1]**.

5.1.1. LAB MEDIA: Figures 3A and 3C
5.1.2. LAB MEDIA: Figures 3A and 3C

5.2. As shown, the pigment cells within the overlaying skin are distributed all over the head **[1]**, interfering with the fluorescent signal in the region of interest **[2]**.

5.2.1. LAB MEDIA: Figures 3A and 3C *Video Editor: please emphasize dark cells in both images*
5.2.2. LAB MEDIA: Figures 3A and 3C *Video Editor: please emphasize green signal in both images*

5.3. After open skull surgery, the area of interest becomes freely accessible for detailed high-resolution imaging **[1]**.

5.3.1. LAB MEDIA: Figures 3B and 3D *Video Editor: please emphasize green signal in both images*

5.4. The skin, skull, and/or blood brain barrier also hinder the penetration of substances into the brain **[1]**, as illustrated by the robust nuclear dye staining observed in these brain cells only after open skull surgery **[2]**.

5.4.1. LAB MEDIA: Figure 3E *Video Editor: please emphasize blue signal*
5.4.2. LAB MEDIA: Figures 3E-3I *Video Editor: please emphasize blue signal in Figures 3F-3I*

5.5. These advantages are also observed at 30 days post fertilization **[1]**.

5.5.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize green signal in Figures 4B-4D*

5.6. In transgenic fish containing a fluorescent brain vasculature due to the expression of the red fluorescent protein mCherry in endothelial cells **[1]**, color coding of the depth

values of the image stack **[2]** demonstrates that even blood vessels deeply buried within the brain to nearly 250 microns are still clearly visible and traceable **[3]**.

5.6.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize red vessels*
5.6.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize figure key*
5.6.3. LAB MEDIA: Figure 5 *Video Editor: please emphasize white vessels*

Conclusion

6. Conclusion Interview Statements

6.1. **Paul Schramm**: When performing this procedure, always be aware that this is a surgery in a living animal and therefore requires respect to the procedure and a focused mind **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.1., 4.2.)

6.2. **Paul Schramm**: After this procedure, recording of the neuronal morphology, including synaptic structures, physiological and intracellular transport events, can also be performed in larvae older than 7 days post fertilization **[1]**.

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

6.3. **Reinhard W. Köster**: Using this method, refinement processes occurring at later larval stages, such as social behaviors or decision making, can also be visualized in vivo **[1]**.

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