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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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KEYWORDS:

zebrafish, *Danio rerio*, in vivo imaging, micro-surgery, open skull, cerebellum, neurons, Purkinje cells, brain, pigmentation

SUMMARY:

Here we present a method to image the zebrafish embryonic brain in vivo upto larval and juvenile stages. This microinvasive procedure, adapted from electrophysiological approaches, provides access to cellular and subcellular details of mature neuron and can be combined with optogenetics and neuropharmacological studies for characterizing brain function and drug intervention.

ABSTRACT:

Understanding the ephemeral changes that occur during brain development and maturation requires detailed high-resolution imaging in space and time at cellular and subcellular resolution. Advances in molecular and imaging technologies have allowed us to gain numerous detailed insights into cellular and molecular mechanisms of brain development in the transparent zebrafish embryo. Recently, processes of refinement of neuronal connectivity that occur at later larval stages several weeks after fertilization have moved to focusced controlling, for example, social behavior, decision making, or motivation-driven behavior. At these stages, pigmentation of the zebrafish skin interferes with light penetration into brain tissue, and solutions for embryonic stages, e.g., pharmacological inhibition of pigmentation, are not feasible anymore.

Therefore, a minimally invasive surgical solution for microscopy access to the brain of awake

zebrafish is provided that is derived from approaches of electrophysiology. In teleosts, skin and soft skull cartilage can be carefully removed by micro-peeling these layers, exposing underlying neurons and axonal tracts without damage. This allows for recording neuronal morphology, including synaptic structures and their molecular contents, and the observation of physiological changes such as Ca^{2+} transients or intracellular transport events. In addition, interrogation of these processes by means of pharmacological inhibition or optogenetic manipulation is feasible. This brain exposure approach provides information about structural and physiological changes in neurons as well as the correlation and interdependence of these events in live brain tissue in the range of minutes or hours. The technique is suitable for in vivo brain imaging of zebrafish larvae up to 30 days post fertilization, the latest developmental stage tested so far. It, thus, provides access to such important questions as synaptic refinement and scaling, axonal and dendritic transport, synaptic targeting of cytoskeletal cargo or local activity-dependent expression. Therefore, a broad use for this mounting and imaging approach can be anticipated.

INTRODUCTION:

Over the recent decades, the zebrafish (*Danio rerio*) has evolved as one of the most popular vertebrate model organisms for embryonic and larval developmental studies. The large fecundity of zebrafish females coupled with the rapid ex utero development of the embryo and its transparency during early embryonic developmental stages are just a few key factors that make zebrafish a powerful model organism to adress developmental questions[1]. Advances in molecular genetic technologies combined with high resolution in vivo imaging studies allowed for addressing cell biological mechanisms underlying developmental processes[2]. In particular, in the field of neuronal differentiation, physiology, connectivity, and function, zebrafish has shed light on the interplay of molecular dynamics, brain functions and organismic behavior in unprecedented detail.

Yet, most of these studies are restricted to embryonic and early larval stages during the first week of development as transparency of the nervous system tissue is progressively lost. At these stages, brain tissue is prevented from access by high resolution microscopy approaches becoming shielded by skull differentiation and pigmentation[3].

Therefore, key questions of neuronal differentiation, maturation, and plasticity such as the refinement of neuronal connectivity or synaptic scaling are difficult to study. These cellular processes are important in order to define cellular mechanisms driving, for example, social behavior, decision making, or motivation-based behavior, areas to which zebrafish research on several weeks' old larvae has recently contributed key findings based on behavioral studies[4].

Pharmacological approaches to inhibit pigmentation in zebrafish larvae for several weeks are barely feasible or may even cause detrimental effects[5,6,7,8]. Double or triple mutant strains with specific pigmentation defects, such as *casper*[9] or *crystal*[10], have become tremendously valuable tools, but are laborious in breeding, provide few offspring, and pose the danger of accumulating genetic malformations due to excessive inbreeding.

Here, a minimal invasive procedure as an alternative is provided that is applicable to any zebrafish

strain. This procedure was adapted from electrophysiological studies to record neuronal activity in living and awake zebrafish larvae. In teleosts, skin and soft skull cartilage can be carefully removed by micro-peeling these layers, because they are not tightly interwoven with the brain vasculature. This allows for exposing brain tissue containing neurons and axonal tracts without damage and for recording neuronal morphology, including synaptic structures and their molecular contents, which in turn include the observation of physiological changes such as Ca^{2+} transients or intracellular transport events for up to several hours. Moreover, beyond descriptive characterizations, the direct access to brain tissue enables interrogation of mature neuronal functions by means of neuropharmacological substance administration and optogenetic approaches. Therefore, true structure function relationships can be revealed in the juvenile zebrafish brain using this brain exposure strategy.

PROTOCOL:

All animal work described here is in accordance with legal regulations (EU-Directive 2010/63). Maintenance and handling of fish have been approved by local authorities and by the animal welfare representative of the Technische Universität Braunschweig.

1. Preparation of artificial cerebro spinal fluid (ACSF), low melting agarose and sharp glass needles

1.1 Prepare the ACSF by dissolving the listed chemicals at following concentrations in distilled water. 134 mM NaCl (58.44 g/mol), 2.9 mM KCl (74.55 g/mol), 2.1 mM $CaCl_2$ (110.99 g/mol), 1.2 mM $MgCl_2$ 6x H_2O (203.3 g/mol), 10 mM HEPES (238.31 g/mol), and 10 mM d-Glucose (180.16 g/mol).

NOTE: For $MgCl_2$, $CaCl_2$, and KCl, 1 M stock solutions are prepared in desalted sterile water and stored at 4 °C for subsequently preparing fresh ACSF. Glucose, HEPES, and NaCl are dissolved as solid compounds in the fresh ACSF solution. For dissolving chemicals, follow the manufacturer's instructions.

1.2 Adjust the pH of the ACSF to 7.8 with 10 M NaOH. Preparation of ACSF requires precise measurement of chemicals and fine adjustment of pH as it replaces the cerebro spinal fluid and maintains the physiological conditions required for neurons to be fully functional else it might cause brain misfunction and neuronal death.

1.3 Store the freshly prepared ACSF at 4 °C for a maximum of 4 weeks. For working conditions, aliquot the required volume of ACSF for the day/experiment and prewarm at 25–28 °C (and optionally oxygenate it, step 2.5)

NOTE: Freshly prepared ASCF is fine for 1 day. If planning to use it over several days, ACSF needs to be sterile filtered.

1.4 For later anesthesia of the larvae, prepare a 50 mM stock solution of d-Tubocurarine in distilled water and store the solution at -20 °C as 100 µL aliquots in the freezer until needed.

1.5 To embed the fish, prepare 2.5% low melting (LM) agarose by dissolving 1.25 g LM-agarose (**Table of Materials**) in 50 mL ACSF and boil until the agarose is completely dissolved.

NOTE: Alternatively, higher or lower concentrations of LM-agarose can be used depending on the experimental set-up. However, if the agarose is too soft, it will not be able to hold the fish in position when opening the skull.

1.6 Store the agarose at 37 °C waterbath, to avoid solidification and because this temperature will also not harm the larvae when embedding. After the boiled agarose is cooled down to 37 °C in the waterbath, add the necessary amount of d-Tubocurarine to the aliquoted agarose needed for the day to reach a working concentration of 10 µM. For future use, store the left-over agarose at 4 °C to avoid contamination.

1.7 Prepare sharp and thin glass needles from glass capillaries (**Supplementary Figure 1**) using a micropipette puller with the following settings.

1.7.1 Puller I, Capillary type 1: Heat 1: 65.8; Heat 2: 55.1; 2-step pulling
Puller II, Capillary type 2: Heat = 700; Fil = 4; Vel = 55; Del = 130; Pul = 55; 1-step pulling.

NOTE: The units are specific for each puller and glass capillary used here, respectively (see **Table of Materials**). Other capillaries and pullers can also be used to prepare the glass needles. But the glass needles should not be too thin as they might break when coming in contact with the skull. Capillary: length: 100 mm (4 inches); OD: 1.5 mm; ID: 0.84 mm; filament: Yes

2. Anesthesia of larvae and preparations for embedding

2.1 When starting the experiment for the day, transfer the animals that are needed with a plastic Pasteur pipette to a 90 mm diameter Petri dish, which is filled with either Danieau (for larvae which are still kept in a Petri dish with Danieau) or water from the fish facility (for larvae which are older than 7 dpf and kept in the fish facility).

2.1.1 When pipetting fish older than 2 weeks, make sure the opening of the pipette is large enough to avoid injuring the fish when transferring them. Do not use a net because it will physically damage especially the younger larvae.

2.2 Add *rotifera* or *artemia* nauplii suited for the size of the larvae kept in the Petri dish, to ensure free access to food and maximum health status of the larvae and to reduce stress.

2.3 For embedding, transfer the selected larvae to a 35 mm diameter Petri dish filled with ACSF. Add the necessary volume of d-Tubocurarine to reach a working concentration/effective dose of 10 µM and wait for a few minutes until the larvae are completely immobilized[11].

NOTE: When the fish grow older or if a faster full anesthesia is needed (under 5 min), it is possible

to increase the concentration of d-Tubocurarine (LD_{50} for mice is 0.13 mg/kg intravenously[12]). It is also possible to use a different anesthetic, such as α-bungarotoxin (working concentration: 1 mg/mL), which has the same effect as curare and also keeps the brain fully active[13]. If a fully active brain is not necessary for the subject of interest, Tricaine in a non-lethal dose (0.02%) is also an option to fully anesthetize the larvae. However, Tricaine blocks sodium-channels, thereby impairing brain activity[14].

2.4 Prepare the mounting chamber by taking the lid of the 35 mm diameter Petri dish, flip the lid upside down, and place a square glass coverslip (24 x 24 mm) on the bottom of the lid. See **Figure 1** (upper part) for a schematic description of these steps. The smoother surface of the glass prevents slipping away of the agarose block, which contains the larvae during the skull opening procedure.

2.5 Aliquot the amount of ACSF needed for the day in an appropriate vial (e.g., 50 mL tube, beaker, schott-bottle, etc.) and oxygenate it with carbogen (5% CO_2, 95% O_2). If imaging only morphology (e.g., flourescence patterns) ACSF is still necessary to ensure the integrity of the brain and that cells are not negatively influenced by osmolarity effects but oxygenation of the ACSF is not needed. This step only needs to be done when full brain activity is necessary for imaging.

NOTE: For optimal oxygen saturation of the medium, add an air stone to the end of the carbogen tube. To guarantee a sufficiently high oxygen level, it is necessary to exchange the ACSF in the imaging chambers with freshly oxygenated ACSF every 20–60 min, depending on the number and age of larvae embedded in the same imaging chamber (e.g., For a single embedded larvae ACSF exchange every hour is sufficient. For six larvae older than 14 dpf embedded in parallel, exchanging ACSF every 20 min is necessary) so plan the necessary amount of oxygen saturated ACSF according to the planned experiment.

3. Embedding of the larvae

3.1 Transfer the fully anesthetized larvae with a plastic Pasteur pipette to the (in step 2.4) prepared mounting chamber. Then, carefully remove the excess medium to avoid dilution of the LM-agarose. All the following steps should be performed under a stereo microscope with sufficient magnification.

NOTE: Tilting the mounting chamber can help to fully remove the medium.

3.2 Proceed immediately to the next step, by adding a sufficiently large LM-agarose drop on top of the larvae (circa 1 mL, depending on the size of the larvae) to protect the animals from drying out and to reduce unnecessary stress.

3.3 Orient the larvae in position before the agarose solidifies. Ensure that the dorsal part of the larvae is directed upward. Also, make sure to embed the larvae as close to the surface of the agarose as possible.

NOTE: Depending on the size and number of larvae planned to embed at the same time, it is possible to adjust the agarose concentration. For example, for 1–3 larvae that are 30 dpf old, a concentration of 1.8%–2% LM-agarose is recommended. For 1–4 larvae that are 7 dpf old, it is most practicable to use 2.5% LM-agarose, whereas, for 5–8 larvae, 2% is more suited. If a fully active brain is required, it is recommended to only embed three fish at the same time to reduce the time needed to operate the larvae. In general, it is recommended to use lower concentrations (1.8%–2%) the older the larvae get or the more larvae are planned to be embedded at the same time.

3.4 If images will be recorded using an inverted microscope, trim the agarose block containing the larvae into a small cuboid shape. This is important for transferring the larvae to the imaging chamber later on. If using an upright microscope, such trimming is not necessary, because the mounting chamber can also be used as the imaging chamber. In **Figure 1** (upper part), one can find a schematic description of these steps.

4. Exposing the brain

NOTE: All the following steps should be performed with greatest care to not unnecessarily injure the larvae. If a fully active brain is required for the experiment, keep in mind that with every second that passes, while the fish is still fully mounted in agarose and has an open skull without oxygenated ACSF, the brain will suffer from a lack of oxygen and also dry out. The effects of oxygen deficiency will become even more dramatic, the older the embedded larvae are. Therefore, it is important to perform the surgery not only within the shortest time possible, but also with maximum precision to not evoke mechanical brain damage with the needle. When trained, steps 4.2–4.4 should not take more than 30 s per fish.

4.1 Begin the surgery as soon as the agarose has solidified. First, trim away all of the excess agarose above the brain region of interest to obtain free access to the head and a clear working space. If the dorsal part of the head is already sticking out of the agarose, skip this step.

4.2 Depending on the region of interest, pick a spot to begin with the surgery. Take the glass needle and make a small incision through the skin but without penetrating too deep into the tissue. This will be the starting point for peeling away the overlaying skin.

NOTE: For optimal results, never start directly above the region of interest to reduce the risk of damaging important structures. If necessary, it is possible to even start posterior to the hindbrain and from there work forward until the unwanted area of skin is peeled away.

4.3 Continue with very small cuts along the part of skin aiming to remove by barely moving the needle just underneath the surface. Most of the time it is not necessary to move completely around the brain and to cut out a circle-like piece of skin and skull, but rather just make two incisions along the head and then push the skin away to one or the other side. **Figure 2** shows a schematic representation of the optimal cutting strategy to obtain free access to the cerebellum.

NOTE: This micro-surgery is a delicate procedure and it will most likely need some training to perfectly remove the skin without damaging the underlying brain. It is also recommended to find out the optimal cutting strategy for the brain region of interest and stick with it for the period of the experiment.

4.4 Immediately after removing the skin from all embedded larvae, proceed by pouring (oxygenated) ACSF over the agarose to flood away unwanted skin particles and blood and to keep the brain fully active and protect it from drying out.

NOTE: If a healthy brain is needed for the experiment, it is recommended to go for a maximum of three fish at a time.

4.5 If using an upright microscope, start directly with imaging.

4.5.1 When using an inverted microscope, slide a small spatula underneath the cuboid agarose block (step 3.4).

4.5.2 Add a small drop of LM-agarose to the bottom of the imaging chamber (e.g., glass-bottom dish) and immediately flip the agarose block containing the larvae with the spatula for 180° and gently push it to the bottom of the imaging chamber, while the liquid agarose drop acts as glue.

4.5.3 When the agarose has solidified, fill up the imaging chamber with (oxygenated) ACSF, then begin imaging. See **Figure 1** (lower part) for a schematic description.

4.6 When full brain activity is required for the experiment, always make sure that ACSF in the imaging chamber has a sufficiently high oxygen level. To ensure this, exchange the medium carefully with freshly oxygenated ACSF when possible every 20–60 min (depending on the number and size of the fish, size and surface of the imaging chamber, and imaging duration).

[place **Figure 1 and 2** here]

REPRESENTATIVE RESULTS:

Figure 3A,C show a 14 dpf larva of the transgenic line tg[-7.5Ca8:GFP][15] with the skull still intact. The pigment cells in the overlaying skin are distributed all over the head and are interfering with the fluorescence signal in the region of interest (here, cerebellum). With the larva in this condition, it is not possible to obtain high resolution images of the brain. **Figure 3B** shows a larva of the same transgenic line after performing the open skull surgery. The area of interest is now freely accessible for the excitation laser and emitted fluorescence light to penetrate the tissue without getting scattered and reflected. Pictures taken of this larva will result in detailed high-resolution images (**Figure 3D**), comparable with images of an isolated brain but with the advantage that the brain is still fully active. Additionally, the brain is not losing any fluorescence intensity that would normally be caused by fixation with para-formaldehyde, as would be the

case for a fixed and explanted brain. In a larvae with an open skull and, therefore, a freely accessible brain with fully active neurons, which still posseses all physiologic connections and receives normal input from the sensory system, it will be also possible to observe short termed events, e.g., synaptogenesis, spine growth, dendritic/axonal migration or local mRNA expression[16] under physiologic conditions. Another advantage of this method is, that it is suited for the application of different pharmacological substances to investigate the effects on the living larva without the diffusion problems caused by the skin and skull or the blood brain barrier, which would normally hinder substances to penetrate the brain (**Figure 3E**). To demonstrate that, a 14 dpf larva with an open skull was incubated with Hoechst-33342 (5 µg/mL in ACSF) for 10 min and then imaged (**Figure 3F–I**). **Figure 4A,B** show 30 dpf old larvae before and after opening the skull. It is the most advanced timepoint tested so far for this method. Even at this developmental stage of the larva, it is possible to obtain high resolution images of single neurons and also subcellular compartments using the method described here (**Figure 4C,D**). These 30 dpf old larvae were not showing activity deficits after 1 h of imaging. This method is also used for electrophysiological patch clamp analysis of 30 dpf old larvae and there is no sign for the loss of neuronal activity in Purkinje cells (yet cells close to the surface are delicate and vulnerable) during the first 60 min of being embedded with an open skull. By regularly inspecting the amount of blood flow, it is possible to validate that the larva is still in a healthy status. This status can be maintained, by regularly exchanging or oxygenating ACSF during long-term image recording. It is therefore fairly simple to validate whether a larva is still suited for functional brain studies. To prove, that no important blood vessels are damaged during the micro-surgery and also to show that this method allows much deeper penetration of the excitation laser light into brain tissue and the reflection-reduced emission of photons, a 10 dpf old larvae of the transgenic strain $Tg(flk1:mCherry)_{y206Tg}$[17] was analyzed by this method. These fish contain a fluorescent vasculature in the brain due the expression of the red fluorescent protein mCherry in endothelial cells. A maximum intensity projection of an image stack covering 245 µm in depth reveals the intricate network of the blood vessels meandering through the mid- and hindbrain (**Figure 5A**). Furthermore, color coding of depth values of the image stack demonstrates that even blood vessels deeply burried in the brain at nearly 250 µm are clearly visible and traceable (**Figure 5B**). **Supplementary Video 1A** shows a healthy brain with sufficient blood supply, while **Supplementary Video 1B** shows brain with compromised blood flow due to the lack of exchange of ACSF and lack of medium oxygenation.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic procedure for the preparation of open skull zebrafish for in vivo imaging in a stepwise manner. The working instructions for the different steps can be found in the graphic itself. Graphic designed by Florian Hetsch and adapted by Paul Schramm.

Figure 2: Detailed schematic representation of the micro-surgery performed to remove pieces of skin and skull above the brain region of interest. The red circle marks the spot where the first cut needs to be made. The red-dotted line delineates the optimal path to cut along with the needle to obtain free access to the cerebellum without damaging it. The green arrow marks the direction in which the excessive skin and skull pieces can easily be pushed away. Make sure to never penetrate into the brain tissue during the whole procedure. After successfully peeling away

the skin, the brain region of interest (here, cerebellum) will be freely accessible for any kind of high-resolution in vivo imaging.

Figure 3: Skin peeling in zebrafish larvae provides optimal access for in vivo brain imaging. Confocal microscopy analysis of 14 dpf old transgenic Tg(-7.5ca8:GFP]bz12Tg larvae. Overview of mid- and hindbrain of non-peeled (**A,C**) and skin-peeled (**B,D**) specimens at 20x and 40x magnification respectively. In the latter, the lack of pigment clearly improves image quality and allows for detailed image recording of fluorescent Purkinje cells in the cerebellum. Subcellular imaging and ease of compound administration to the brain is demonstrated by a 10 min incubation period with the nucleic acid fluorescent staining using Hoechst-33342 (5 µg/mL in ACSF). While only skin cells incorporate this dye in non-peeled larvae (**E**), specimens with lifted skin display intense labeling of their nuclei throughout the brain (**F**) allowing for visualizing individual nuclei (**G**) of GFP-fluorescent Purkinje neurons (**H**, merged image shown in **I**, 63x optical and 4x digital magnification). Scale bars: A–F: 100 µm, G–I: 5 µm. Larvae were embedded with their head to the left, dorsal is up.

Figure 4: Skin peeling extends access for in vivo brain imaging to juvenile zebrafish. Confocal microscopy analysis of 30 dpf old transgenic Tg(-7.5ca8:GFP]^{bz12Tg} juveniles. Overview over mid- and hindbrain of non-peeled (**A**) and and skin-peeled specimens at 10x (**B**) and 40x (**C**) and 63x optical magnification with 4x digital zoom (**D**), respectively. The removal of pigmented skin enables visualization of the cellular organization of the continuous cerebellar Purkinje cell layer and to depict projections of individual neurons (**D**, white arrows). Scale bars: A–C: 100 µm, D: 5 µm. Larvae were embedded with their head to the left, dorsal is up.

Figure 5: Skin removal permits brain vasculature reconstruction at extended depth in a 10 dpf old Tg(flk1:mCherry)^{y206Tg} zebrafish larva. (**A**) Maximum intensity projection of a stack of images (245 images of a thickness of 1 µm at total distance of 245 µm, 20x optical magnification) displays a continuous network of blood vessels throughout these brain areas. (**B**) Depth color coding reveals that this network of blood vessels can be monitored at depth of 200 µm and beyond. To remove autofluorescence and reflection, the eyes were masked by black color. Scale bar: 100 µm. Larvae were embedded with their head to the left, dorsal is up.

Supplementary Figure 1: Needles made from glass capillaries. (**A**) Two needle types obtained from two different needle pullers are shown. It is important that the tip is not too long to provide it with sufficient stability and to avoid their breaking when coming in contact with the skin/skull. Yet a sufficient sharpness is needed to allow for making cuts with clean edges. (**B**) An image of the capillary that are loaded in the needle pullers is shown. Scale bar is 250 µm. Capillary length: 100 mm; OD: 1.5 mm: ID: 0.84 mm; filament: yes.

Supplementary Video 1: **Imaging 7dpf old larva.** (**A**) Real-time Movie recording (30 Fps) from a 7 dpf old larva 10 min after the skin above the brain has been removed. The vivid blood flow indicates a healthy state with sufficient blood supply. (**B**) Real-time Movie recording (30 Fps) from the same 7 dpf old larva shown in Supplementary Video 1A, 2 h after the microsurgery without exchange of ACSF and lack of medium oxygenation. Scale bar: 100 µm.

DISCUSSION:

The presented method provides an alternative approach to brain isolation or the treatment of zebrafish larvae with pharmaceuticals inhibiting pigmentation for recording high resolution images of neurons in their in vivo environment. The quality of images recorded with this method is comparable to images from explanted brains, yet under natural conditions.

Furthermore, a loss in intensity of fluorescence is avoided, because there is no need for treatment with fixatives[18]. Also, this method is not as invasive as isolating a brain and it only consists of one crucial step that could lead to failure, so the chances of a successful preparation are higher compared to brain explantation. The most important advantage is, that this method is suited for in vivo image recording. Provided that the skull opening is performed precisely, fast and successful, and oxygen saturation of ACSF is monitored regularly, the brain should be fully functional and viable for hours. Based on electrophysiological recordings, blood circulation, breathing, and survival of fish for additional days, we consider it likely that skin and skull removal is not majorly detrimental to overall health. Thus, this method can be used for real-time imaging of cellular and subcellular processes in a healthy and functional brain without image-disturbing pigmentation. This method is a standard procedure used for electrophysiological recordings of neuronal activity in vivo in our lab and is also well established in other electrophysiology laboratories around the world[19-22], proving that the function of the brain is not impaired by the micro-surgery. One disadvantage that needs to be mentioned is that this approach is only suited for imaging of dorsal and lateral brain regions and cannot be performed repeatedly with the same specimen.

However, if a fully functional brain is necessary for the experiment, the oxygen saturation should be monitored permanently, and, if required, the ACSF in the imaging chamber should be exchanged with freshly oxygenated ACSF. This means, for long-term recordings, there needs to be an option to exchange the used ACSF with oxygen-saturated ACSF without disturbing the imaging-procedure which could be addressed using a miniature pump.

Of note, if the micro-surgery is not performed correctly or imprecisely, it will lead to brain injury or cerebral hemorrhage resulting in compromised brain physiology. Also, a low oxygen saturation in the ACSF (see **Supplementary Video 1**) or too much stress for the larvae during this whole procedure can negatively impact the brain[23,24]. Therefore, this method is not suited for high-throughput analysis, but rather for longitudinal or highly detailed image recording of only a few larvae per timepoint. Therefore, this method should not be aimed for drug screening at high throughput, but it could be used to characterize or validate promising pharmacological compounds in detail, where high resolution imaging is informative and necessary. To ensure proper brain health status, it is recommended to not exceed the number of three larvae per trial.

Through the open skull, it is even possible to directly apply substances to the brain tissue without having them to pass through the blood-brain barrier. Overall, the method described here—currently applied for in vivo electrophysiology—is a technique with a high potential in a very

broad field of in vivo brain imaging and optogenetics in zebrafish larvae and juveniles and can be coupled with neuropharmacological approaches.

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DISCLOSURES:

The authors have nothing to disclose.

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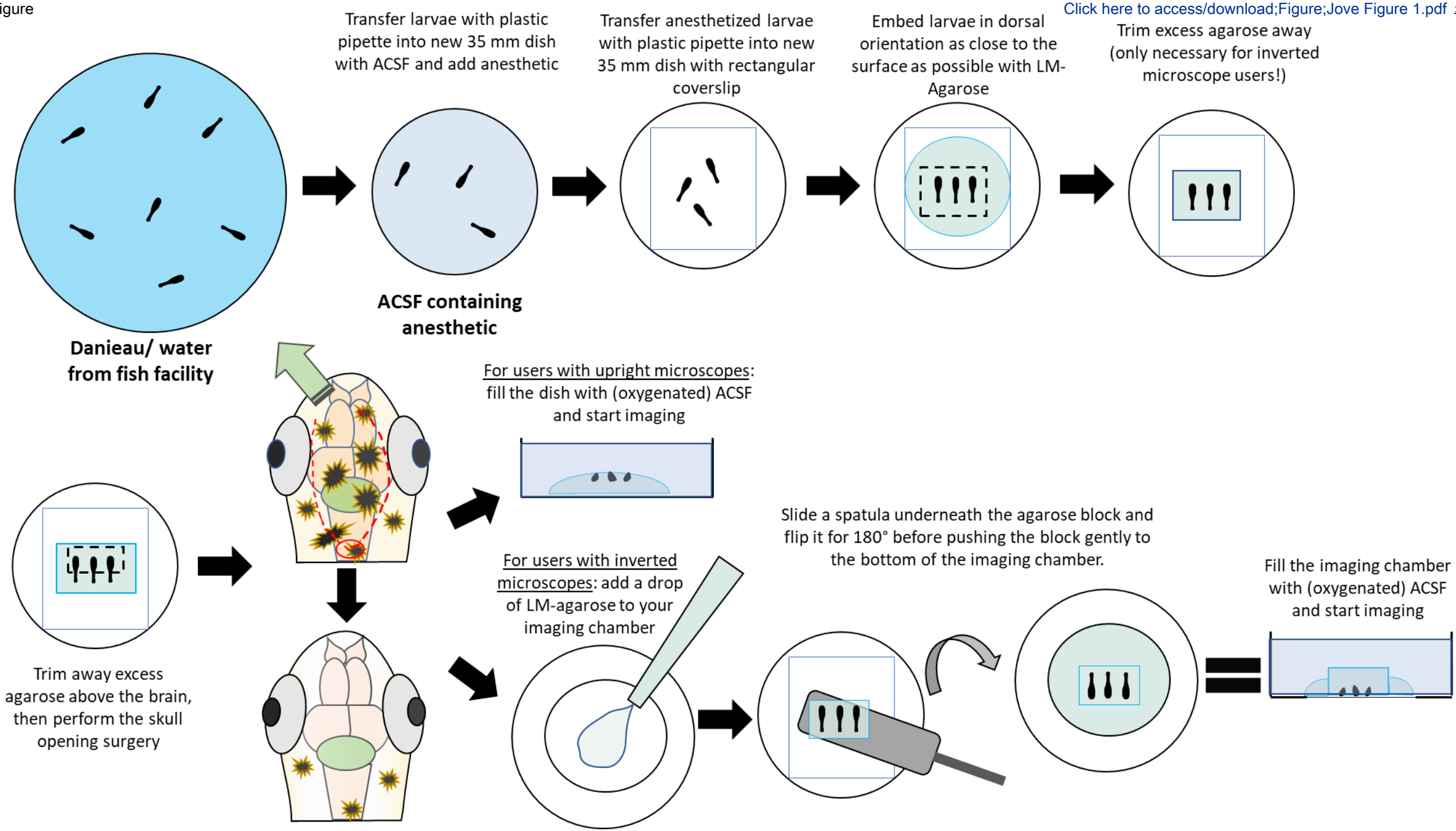
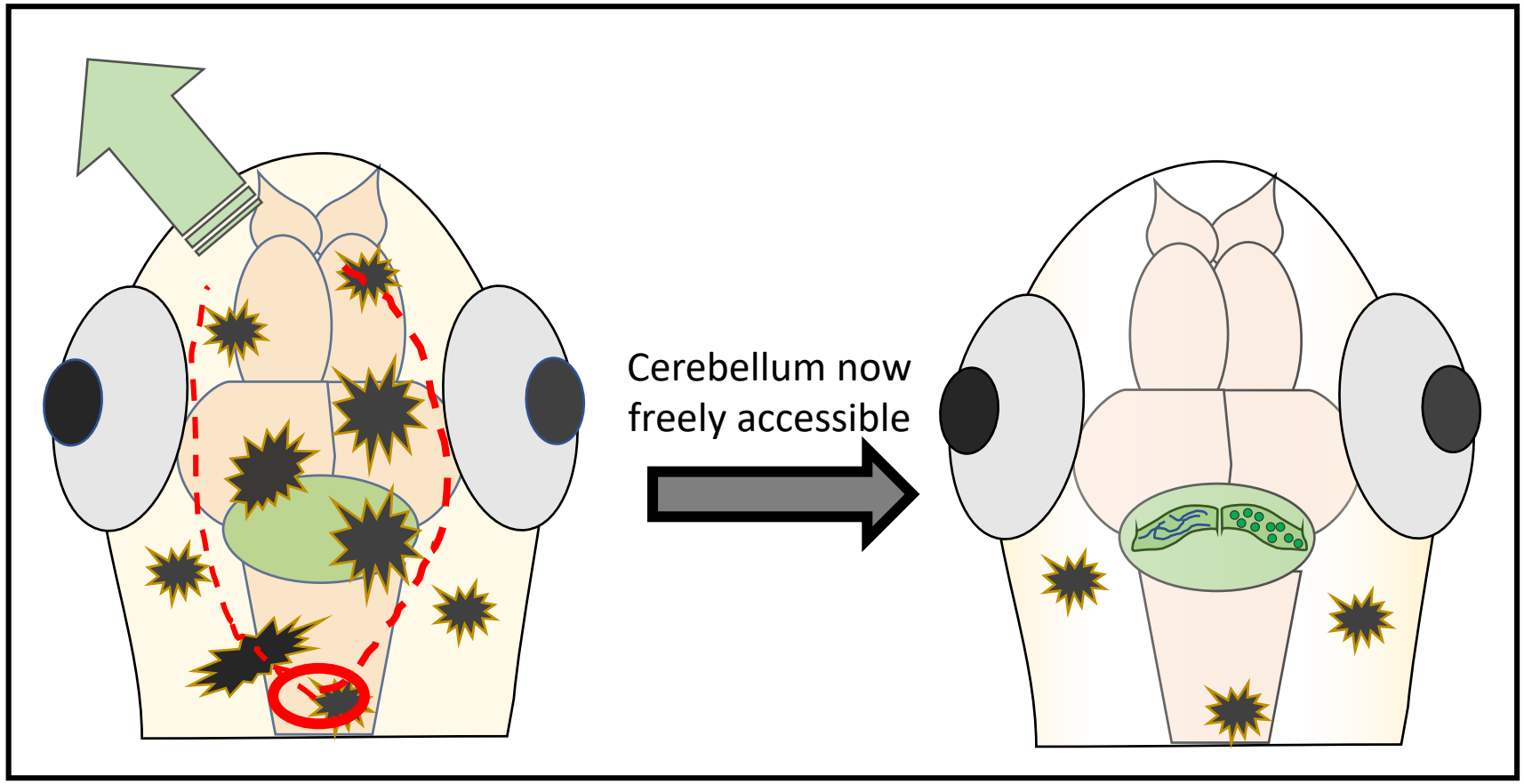
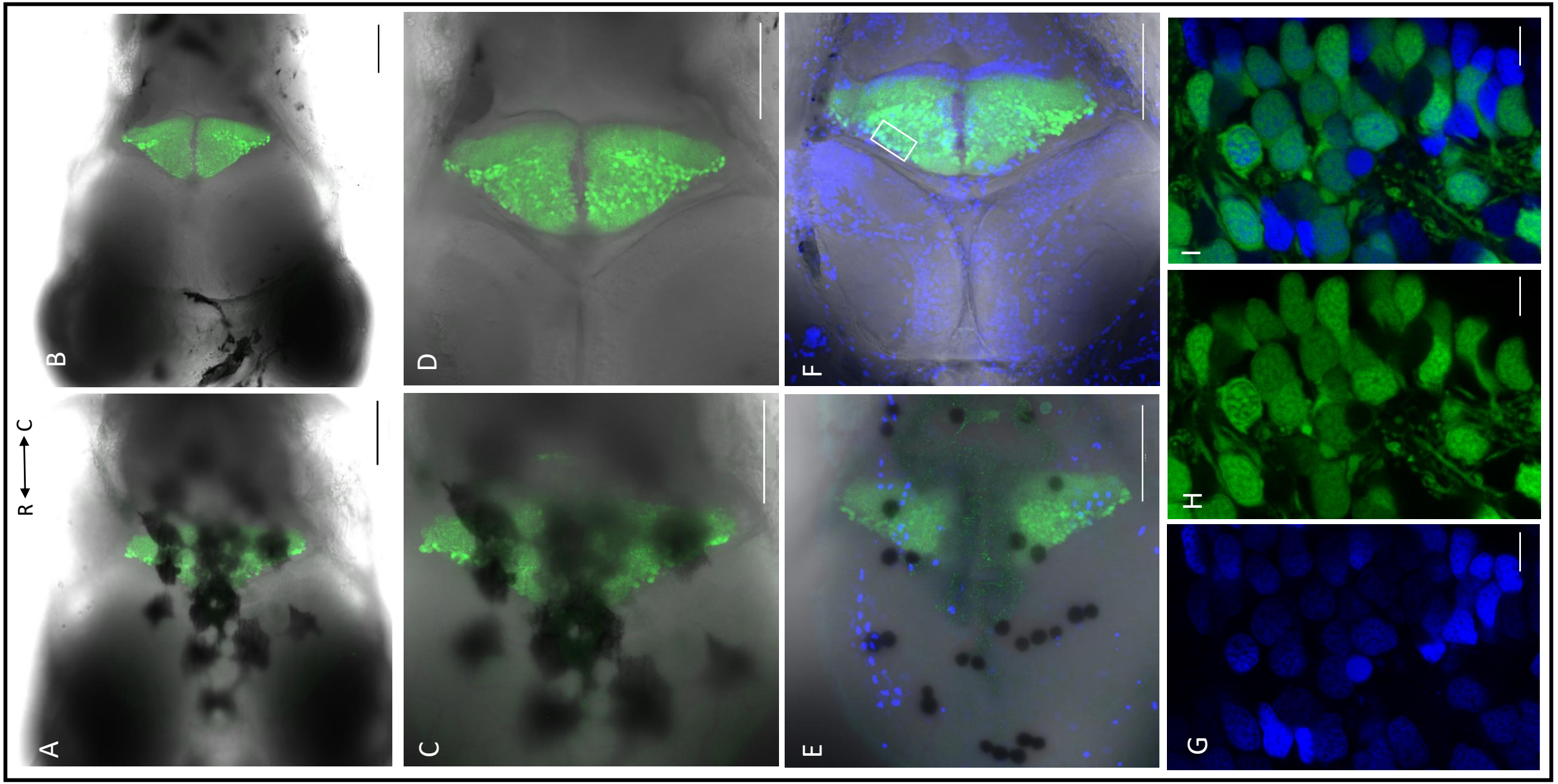
Figure



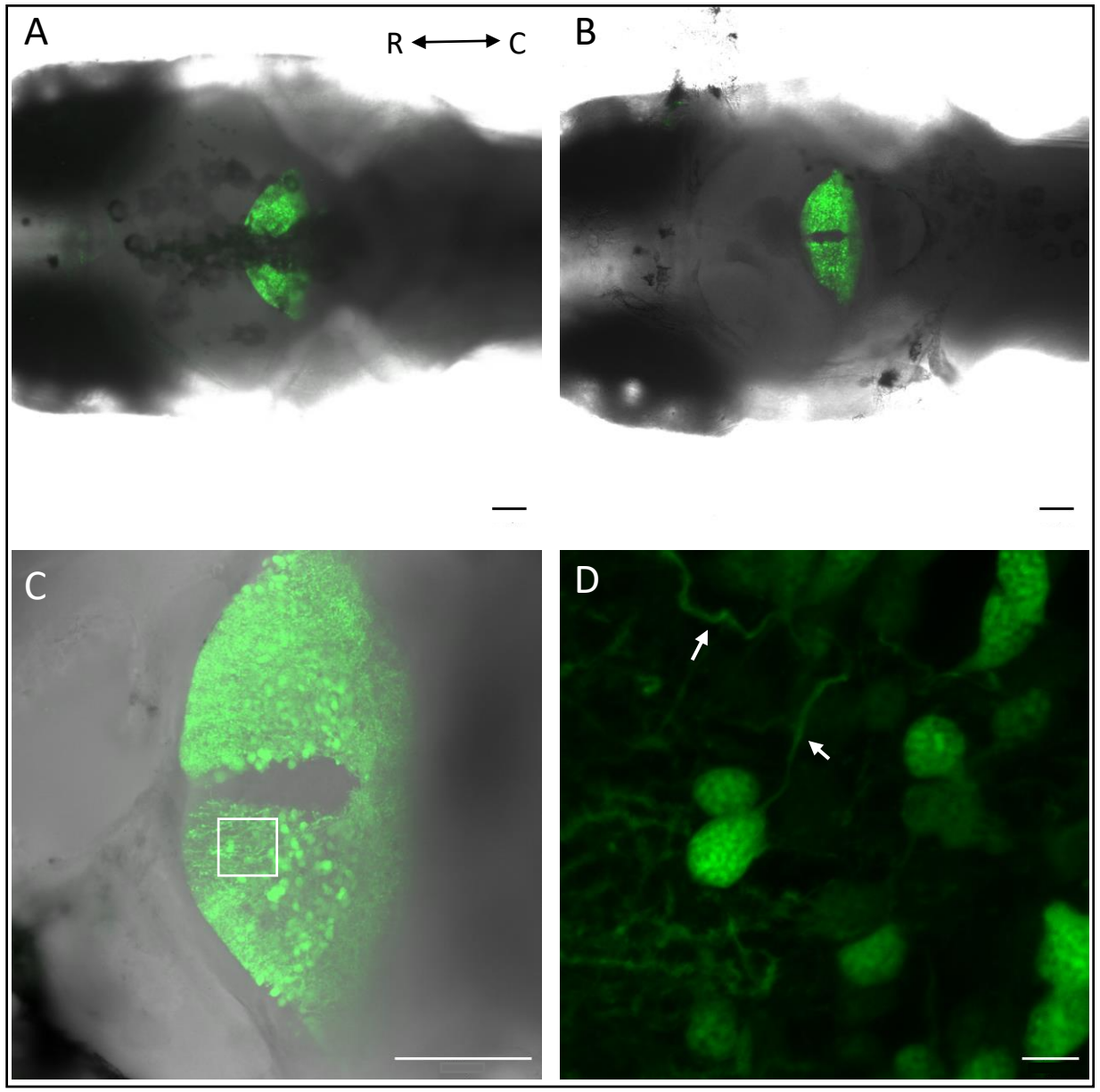
Figure





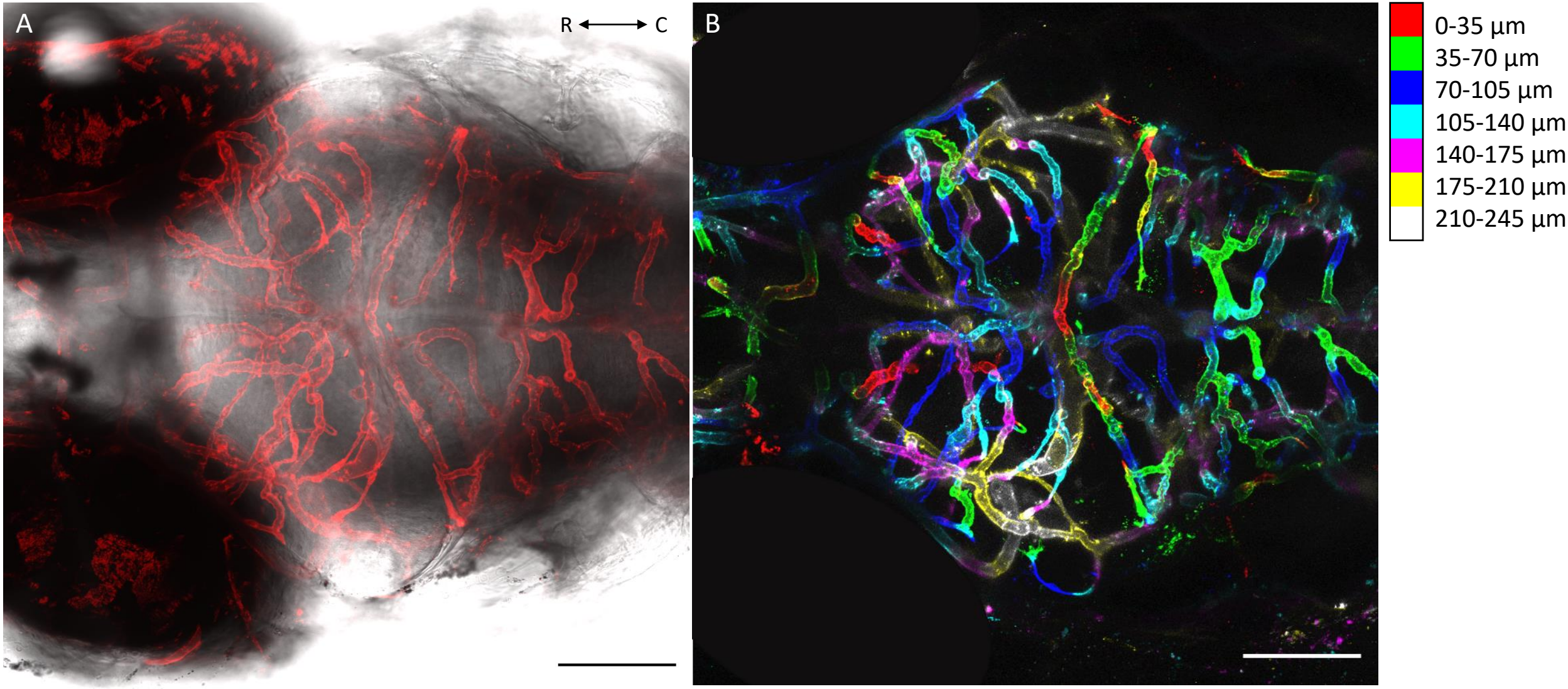
Figure

Click here to access/download;Figure;Jove Figure 4 with arrows in D and R-C axis.pdf



Figure

Click here to access/download;Figure;Jove Figure 5 mit R-C axis.pdf



Supplementary Video 1A

Click here to access/download

Video or Animated Figure

Supplementary video 1A healthy Blood flow final version.mp4

Click here to access/download

Video or Animated Figure

Supplementary Video 1B compromised Blood final version.mp4

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Calciumchloride	Roth	A119.1	
Confocal Laser scanning microscope	Leica	TCS SP8	
d-Glucose	Sigma	G8270-1KG	
d-Tubocurare	Sigma-Aldrich	T2379-100MG	
Glass Capillary type 1	WPI	1B150F-4	
Glass Capillary type 2	Harvard Apparatus	GC100F-10	
Glass Coverslip	deltalab	D102424	
HEPES	Roth	9105.4	
Hoechst 33342	Invitrogen (Thermo Fischer)	H3570	
Imaging chamber	Ibidi	81156	
Potassiumchloride	Normapur	26764298	
LM-Agarose	Condalab	8050.55	
Magnesiumchloride (Hexahydrate)	Roth	A537.4	
Microscope Camera	Leica	DFC9000 GTC	
Needle-Puller type 1	NARISHIGE	Model PC-10	
Needle-Puller type 2	Sutter Instruments	Model P-2000	
Pasteur-Pipettes 3ml	A.Hartenstein	20170718	
Sodiumchloride	Roth	P029.2	
Sodiumhydroxide	Normapur	28244262	
Tricain	Sigma-Aldrich	E10521-50G	
Waterbath	Phoenix Instrument	WB-12	
35 mm petri dish	Sarstedt	833900	
90 mm petri dish	Sarstedt	821473001	

Editorial comments:

Changes to be made by the Author(s):

Editor: 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Authors: Thanks a lot for pointing out these editorial improvements. We have thoroughly read and corrected the manuscript to the best of our knowledge.

Editor: 2. Please change your title to "In Vivo Imagine of Fully Active Brain Tissue in Awake Zebrafish Larvae and Juveniles by Skull and Skin Removal".

Authors: We have implemented this change, the manuscript title has been adapted as suggested.

Editor: 3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Authors: Thanks a lot for clarifying this point to us. We have removed the use of personal pronouns throughout the manuscript.

Editor 4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: NARISHIGE; Sutter Instruments etc

Authors: We have removed all commercial terms, yet according to the instructions of JoVE we have listed the used material with the names of the companies where this equipment or material has been purchased.

Editor: 5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Authors: Thanks a lot for this clarification. We have carefully adopted this imperative style and hope that the manuscript now reads according to the editorial style of JoVE.

Editor: 6. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

Authors: We have implemented all these editorial suggestions.

Editor: 7. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.

Authors. We have corrected our list of references according to these guidelines and hope to completed these requirements adequately.

Editor: 8. In Figure 1, please change "larvaes" to "larvae" and "anaesthetic" to "anesthetic" (American English spelling).

Authors: We have carefully read through our manuscript again. We have changed all questionable spelling to American English.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript titled "protocol for In vivo imaging of fully active brain tissue in awake zebrafish larvae and juveniles by removing parts of the skull and skin" by Schramm et al provides an overview of how to perform microsurgery to remove the skin and skull for the proper visualization of the brain tissue in zebrafish larvae and juveniles. The authors have demonstrated the surgical procedures involved in exposing the live brain tissue, both in descriptive and illustrative ways. All the advantages and the limitations of this protocol are also pointed out by the authors. The authors are clear in the goals of their protocol. Overall, this protocol provides a method for the electrophysiological study of the neuronal activity in a morphologically intact zebrafish beyond 7-8 dpf larvae after which it is difficult to maintain the transparency of zebrafish larvae.

Authors: We cordially thank the reviewer for having read our manuscript so carefully and the motivating judgement.

Reviewer1: Major Concerns: I do not see any major concerns with the manuscript. Most of the things regarding the drawbacks and advantages of this method has been discussed by the authors in the discussion section.

Authors: We are happy to hear that the reviewer considers our described method and manuscript as solid.

Reviewer1: Minor Concerns: My only minor concern is that the authors have written that this protocol could be used for the neuropharmacological purpose while also acknowledging that this protocol cannot be used in high-throughput methods.

Authors: The reviewer is right in saying that the method is not suitable for high throughput approaches. What we wanted to point out is that this method can be used to characterize or validate pharmacological compounds and that this approach is easy to perform with the direct access to the brain. We consider it feasible to study five compounds per day, but clearly this is not high throughput. We have therefore changed the manuscript accordingly and thank the reviewer for having pointed out this misunderstanding to us.

Reviewer #2:

Manuscript Summary: With some exceptions, in vivo imaging of the zebrafish CNS has largely been done at early developmental stages. The protocol that Schramm and colleagues report here allows for in vivo imaging at later stages, circumventing problems with pigmentation and permitting light microscopy based imaging of brain structures at greater depths.

Reviewer2: Major Concerns: None

Authors: We are delighted that the reviewer is convinced about the method that we have introduced here.

Reviewer2: Minor Concerns: Abstract - line 47 Should be: Therefore, we provide a minimally invasive….

Authors: We have corrected this point.

Reviewer2: 1.2 ….else it will cause brain….(sounds too conversational)

Authors: We have changed the wording to “might case”.

Reviewer2: 1.1 Would it be worthwhile to sterilize the ACSF? It would also be good to indicate the molecular weight for each chemical.

Authors: Yes, the reviewer is right about that. Sterile filtering would be a good option. We have now added this possibility to the manuscript and thank the reviewer for this valuable thought.

Reviewer2: 1.6 It would be worth providing an image of the glass capillary - to see for example how long the shank is. An image of a pulled and un-pulled capillary for comparison with a scale bar would be helpful.

Authors: We thank the reviewer for this helpful advice. We now display a picture of the capillary as suggested.

Reviewer2: 1.6.1 Should be Heat not Heater - and presumably 65.8 not 65,8.

Authors: The reviewer is right and we have changed the text accordingly.

Reviewer2: 2.3 How are the stock solutions for the drugs prepared? Are the manufacturer's instructions followed or are there deviations worth mentioning?

Authors: For dissolving all chemicals manufacturer's instructions were followed. In detail, $MgCl_2$, $CaCl_2$ and KCl 1M stock solutions were prepared in desalted sterile water for subsequently preparing fresh ACSF. Glucose, HEPES and NaCl are dissolved as solid compounds in the fresh ACSF solution. D-Tubocurare is diluted in water at a concentration of 50mM and stored as 100µl aliquots at -20°C. To prepare 2.5% low melting agarose solutions, 1g of low-melting agarose was dissolved in 40ml freshly prepared ACSF. All these procedures are detailed in the manuscript to allow for reproducing these preparations.

Reviewer2: 2.4 The glass coverslip is square - 24x24 mm - rather than rectangular. Can the authors say why the larvae are embedded on the glas coverslip and not directly on the plastic lid. Is the coverslip attached to the plastic lid in some way?

Authors: The glass coverslip acts as operation table for the skull opening procedure. The surface of the glass provides the agarose a stronger hold during the surgical procedure and prevents the larvae from slipping away. In addition, the subsequent transfer of the agarose block is easier to perform from a glass bottom surface compared to plastic. We are mow mentioning this advice in the manuscript and thank the reviewer for pointing out to us these helpful clarifications.

2.5 What is aquaristic? Aquatic tanks?

Authors: We meant aquatic tanks, but we have left out this term completely in the revised manuscript as it does not provide additional helpful advice.

Reviewer2: 2.5 By 'full brain activity' - are the authors referring to calcium imaging experiments? If ACSF is not necessary for imaging 'only morphology' what medium is preferred? Would it be detrimental to have ACSF if only morphology is being imaged?

Authors: The reviewer is right the mere image recording at individual stages is not as challenging in terms of media used, here the surgery should be performed in the presence of ACSF and fish larvae can be fixed PFA subsequently. Live imaging for example to image Ca^{2+}-transients requires ACSF for oxygenating the brain tissue sufficiently. This also ensures that neuronal morphology is not altered due to osmolarity effects. We have added these points to the manuscript to discriminate properly between the different purposes and media to be used.

Reviewer2: 2.5 Note - exchanging the ACSF every 20-60 mins is a very broad window of time. Would it not be better to keep this shorter?

Authors: We now provide more details about this point in the manuscript. The period of ACSF exchange depends on the number of larvae embedded within the same dish. For single embedded larvae ACSF exchange every hour is sufficient. For six larvae imaged in parallel, ACSF exchange should occur every 20 minutes. We hope we have now clarified this point sufficiently.

Reviwer2: 3.3 Line 192 - make clear that 'as close to the surface..' refers to the agarose.

Authors: We have clarified this point in the manuscript.

Reviewer2: Line 199 - unclear what is meant by 'ensure a fast brain exposure for all larvae'. Do the authors wish to simultaneously image all 3 brains at high speed?

Authors: We have rephrased this sentence for clarification to “reduce the time needed to operate larvae…”

Reviewer2: 4.1 - 4.3 represent the most important steps of the protocol and will presumably be filmed in good detail. The description is good but I think an experimenter would greatly benefit from seeing the procedure being done - Figure 2 essentially shows a 'before' and 'after' scenario and thus does not (and cannot) show the microsurgery.

Authors. We thank the reviewer for pointing this out and we will make sure that video sequences are provided for this experimental step.

Reviewer2: 4.5 Presumably the imaging chamber for use at an inverted microscope is a glass-bottom dish? Or a plastic petri dish in which a hole is cut out to fit the glass coverslip, with the agarose-mounted fish?
Figure 3 very beautifully illustrates how the microsurgery provides access for microscopy!

Authors: The reviewer is right, the imaging chamber is a glass bottom dish, we prefer to use the 35mm µ-dish from Ibidi Inc, but it can be easily self-made as described by the reviewer. We now mention this in the revised manuscript.

Reviewer2. Line 269 excitation laser rather than exciting laser.

Authors. The reviewer is right and we changed the text accordingly.

Reviewer2: Line 274 -276 'In a freely accessible brain….' It is unclear whether the authors are equating a fixed brain with an explanted brain (the latter would be considered ex vivo and still considered 'alive' for a period of time).

Authors: We changed this sentence to clarify the advantages of the open skull method. The major advantage compared to an explanted unfixed brain (ex vivo) is, that the brain in an open skull fish still contains all the neuronal connections and receives normal sensory input from all sources and therefore processes like synaptogenesis should occur under normal physiological conditions. In an explanted brain, the physiologic input is significantly altered because all the circuitry which connect the brain with the rest of the body are removed.

Furthermore, it is very challenging to explant a brain from a larva between 4-30 dpf without fixing the fish for at least 5-10 minutes in PFA, because the tissue is incredible sensitive to mechanical influence, that's why we equated the fixed brain with an explanted brain. We hope we sufficiently clarified this comparison in the revised version of the manuscript.

Reviewer2: Line 292 - should be prove - not proof

Authors: We have adjusted the text accordingly.

Reviewer2: Line 338 - no white arrows can be seen in 4D

Authors: We have added this arrow to the figure.

Reviewer2: Figure 1 - Top left part of the schematic mentions Danieau. 2.1 however only mentions water from the fish facility (are there instances when Danieau would be the preferred medium?). It should be 'larvae' not 'larvaes' - check throughout labeling of Fig 1.

Authors: Danieau is used as rearing medium up to 7dpf, subsequently water from the fish facility will be used as fish are adapted to this environment.

Reviewer2: Figures 3-5: Indicate the rostro-caudal axis. Scale bar numbers are too small.

Authors: All fish were embedded with their head to the left, dorsal is up according to the convention. But to avoid misunderstandings we have clarified this in the image description. The scale bars have been adjusted and the numbers were removed from the images themselves and can now only be found in the image description. An arrow indicating the rostro caudal orientation has been added.

Reviewer2: Table of Materials: Kalium chloride should be potassium chloride

Authors. We have changed this misspelling accordingly.

Reviewer2: Discussion: How long can the brain in this procedure be kept alive. The authors mentioned no decline in calcium activity in 30 dpf larvae after 1 hour of imaging - is this then the upper limit?

Authors: Based on electrophysiological recordings we see no change in activity for the first two hours. But as we record from the cerebellum, we did not want to extrapolate this too extensively to other brain areas and considered 1 hour as a conservative estimate. After the surgery of 7 dpf larvae, they survived for more than 48 hours and also started to successfully hunt prey (e.g. *Rotifera*) and could swim normally and reacted to stimuli (e.g. tapping of the petri dish). Currently we do not know if it is possible to raise these larvae to adulthood, but at based on the feeding behavior it seems that the surgery had no gross influence on the health of the larvae. Nevertheless, we have toned down our statement (please see also our answer to reviewer 3).

Reviewer #3:

Manuscript Summary: This method to study the live larval zebrafish brain from Schramm et al. will be a useful addition to the field. The procedures are generally explained well. Of course, the utility of the technique will be enhanced by providing videos, which are not available for this reviewer to evaluate. Other than this drawback, toning down some speculative aspects and providing additional technical details (noted below) will improve the manuscript and make it suitable for publication.

Reviwer3: Major Concerns: L136-142: While instrument settings are useful, the authors should also provide a technical description of the glass pipets. Tip diameter, shank length (short or long) etc.

Authors: The reviewer is right in pointing this out to us. We have added this information to the revised manuscript and we now also provide a picture of the capillary.

Reviewer3: L155-157: Is d-tubocurarine just added to the fish water? Is it able to penetrate the skin and have consistent and strong effects?

Authors: The reviewer is right, d-Tubocurarine is directly added to the ACSF. The anesthetic is absorbed through the mucous membrane of the fish skin and also by breathing through the gills, when the larvae are older than 7 dpf. The time that is needed for a complete anesthesia depends on the age of the larvae and also of the concentration of the anesthetic, but we provide

concentations and time points for orientation. d-tubocurarine has a strong anesthetic effect for all timepoints tested (4-30 dpf) and should theoretically also be able to anesthetize adult zebrafishes.

Reviwer3: L219-240: It would be very helpful to view Videos for steps 4.1 to 4.3, without which these descriptions are not sufficiently detailed

Authors: We agree with the reviewer and marked these steps for video recording.

Reviewer3: L213-214: What are the signs of oxygen deficiency in anesthetized larvae?

Authors: Declining oxygen levels can be observed by a decline in heart rate and blood flow through the vasculature and should be monitored regularly. We now provide exemplary movies in the supplementary material of our revised manuscript.

Reviewer3: L238-240: This technique will work quite well for imaging in the tectum, cerebellum and the hindbrain because it is relatively easy to peel the skin due to the large 4th ventricle, and the orientation of the tissue. Have the authors attempted peeling the skin more anteriorly, and how to image?

Authors: Yes, we also tried to peel away skin in telencephalic regions to obtain access to more anterior regions of the zebrafish brain to investigate for example cells in the pallium. Here, peeling away skin should start near the mouth with the help of incisions directed dorsally around the eyes. Only the required region of interest should be exposed to reduce the risk of rupturing blood vessels. It will help to mount the larvae in a tilted way with the mouth pointing to the dorsal surface of the agarose. The procedure requires thorough training for the forebrain compared to the cerebellum or the posterior optic tectum as these are positioned most dorsally and easily accessible, yet imaging forebrain cells has worked for us successfully.

Reviewer3: L241-242: There is no d-tubocurarine in the ACSF. In its absence, doesn't its paralytic effect disappear after some time due to dissociation from the receptors? Why not include d-tubocurarine in the ACSF?

Authors: The reviewer is right and we forgot to mention this point in the manuscript. D-Tubocurarine is added to the LM-agarose once it has cooled down and shortly before mounting larvae at an effective concentration of 10µM, here the agarose also serves as a reservoir for the anesthetic. We have added this important information to step 1.6 in the revised manuscript.

Reviewer3: L299-300: While this experiment suggests that skin and skull removal is not detrimental to health, it is hard to judge without comparing to an unoperated sibling. While the vasculature is intact, one cannot conclude its condition (morphology and distribution) is normal without comparing to a control.

Authors: The reviewer is right and we have decided to tone down this claim in the manuscript by stating now “based on electrophysiological recordings, blood circulation, breathing and survival of fish for additional days we consider it likely that skin and skull removal is not majorly detrimental to overall health”. We consider this statement as appropriate, as we cannot see morphological changes of the vasculature and no obvious bleeding but rather a vivid blood circulation. The electrophysiological activity of cerebellar neurons is unaltered during the first hours and compares well to published data. After experimental analysis zebrafish larvae swim and feed, and it is possible to keep larvae alive for several days after the surgery, where we observe them to swim and feed.

Reviewer3: L338: Arrows missing in Fig. 4D

Authors: We have added the missing arrow to the respective figure.

Reviewer3: L362-363: There is no evidence presented for healthy brains after 1-2 hours. This statement (brain is fully functional and viable for hours) is speculative, and would be warranted only if the suggested controls have been performed. It does not appear that any of the papers cited (#s 19-22) conducted long-term observations on operated larvae beyond 1 hour.

Authors: Based on our own electrophysiological recordings we see no change/decline in electrophysiological activity for the first two hours, and these data correlate well to previously published electrophysiological signatures of cerebellar neurons. But as we record from the cerebellum, we did not want to extrapolate this too extensively to other brain areas and considered 1 hour as a conservative estimate.

As this method focuses on brightfield and fluorescence imaging we do not want to dilute the focus by providing electrophysiological recordings, but we now mention these findings in the discussions to clarify the experimental base for this claim. If the reviewer wishes, we can provide these data for the purpose of the manuscript review, but would like to avoid to present a mixture of techniques in the method protocol.

Of note, it is possible to raise 7 dpf old zebrafish larvae after skull opening for more then 48 hours. Their movements seem to be normal, they start to hunt prey (e.g. *Rotifera*) and are able to feed and to react to external stimuli (e.g. tapping on the petri dish). Currently, we do not know if it will be possible to raise these larvae to adulthood, but there are no gross behavioral or morphological deficits observable by us in these larvae.

Reviewer3: Minor Concerns:

Reveiwer3: L174: change "aquaristic" to "aquarium"

Reviewer3: Figure 1: Change "larvaes" to "larvae", which is the plural of larva.

Reviewer3: L291-292: Change to "To prove that"

Reviewer3: L386: Change "the here described method" to "the method described here"

Authors: We have made all suggested corrections to the wording in the revised version of the manuscript.

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