

Chapter 14

Anatomical Dissection of Zebrafish Brain Development

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Abstract

Zebrafishbrain.org is an online neuroanatomical atlas of the embryonic zebrafish. The atlas uses high-resolution confocal images and movies of transgenic lines to describe different brain structures. This chapter covers detail of materials and protocols that we employ to generate data for the atlas.

Key words Zebrafish, Neuroanatomy, Brain atlas, Brain dissection, Labeling

1 Introduction

Increasingly zebrafish are becoming a model system for the study of behavior [1–5]. To truly understand zebrafish behavior, we must first understand the connectivity of the neuronal circuits driving behavior. This requires detailed neuroanatomical characterization of the zebrafish brain. Zebrafish are a relatively new model system; as such, the zebrafish does not benefit from the extensive neuroanatomical descriptions that have been undertaken in other model species [6, 7]. Most neuroanatomical studies in the zebrafish and other teleosts focus on adult neuroanatomy [8, 9]. These studies mainly use serial sections of adult brains which can be challenging to interpret by non-experts and can be difficult to apply to the embryonic brain [10]. Specific description of the neuroanatomy of the embryonic and larval zebrafish brain is not comprehensive. To address this issue, in collaboration with other laboratories, we are in the process of building an online neuroanatomical resource (an atlas) called zebrafishbrain.org.

Zebrafishbrain.org is being built to communicate current knowledge about the neuroanatomy of the developing zebrafish brain, and this is achieved in two ways. The first is to provide a hub for community experts to provide data and write descriptions of neuroanatomical structures, and the second is to provide data and descriptions ourselves. To do this we are mining currently available collections of transgenic zebrafish (and making a few of our own) to generate a

collection of transgenic lines with thoroughly described embryonic/larval (and sometimes adult) brain-specific expression patterns. We employ the descriptions of the expression patterns of these lines in the construction of tutorials describing brain structures.

This chapter details the core methods that are currently employed by us when studying transgenic zebrafish embryos and larvae for zebrafishbrain.org. The protocols describe in detail how we undertake brain dissection, immunohistochemistry, and the several available methods we employ to mount embryos for confocal microscopy. We also list software we have found useful for processing the raw data.

The protocols are based upon methods that are widely used in the zebrafish field but with a few adjustments to adapt to characterization of zebrafish developmental neuroanatomy. We also specify some reagents that we have found to be reliable for use in the zebrafish. Many of these protocols have flexible aspects to them, and we have tried to include detail of this flexibility where possible as an indication of how optimization for particular conditions can be undertaken.

2 Materials

2.1 Equipment for Embryonic Culture and Fixation

1. Petri dishes.
2. Watchmakers forceps for dechoriation: Dumont #5 (Fine Science Tools).
3. 7 ml bijoux tubes: Appleton Woods.

2.2 Embryonic Culture and Fixation Reagents

1. Fish/embryo water (filter-sterilized aquarium system water) or embryo medium (zebrafish book).
2. 1-Phenyl 2-thiourea (PTU) 3 mg/ml: Keep frozen 40 ml aliquots of 25× stock solution (75 mg/ml). One aliquot makes 1 l of PTU when mixed with fish water. Fresh PTU should be made every 2 days.
3. Sweet fix: 4 % PFA with 4 % sucrose. Make from 20 % PFA stock: 40 ml H₂O + 10 g paraformaldehyde. Heat to 60 °C with stirring. Add 10 drops of 10 M NaOH. Cool and filter through funnel with filter paper, add 10× phosphate-buffered saline (PBS) (pH 7.3), sucrose, and H₂O and adjust pH with HCl to give a final concentration of 4 % paraformaldehyde, 4 % sucrose, and 1× PBS at pH 7.3. Aliquot into 5 ml aliquots and freeze. Defrost fresh fix at room temperature (RT) just prior to fixation. Do not use fix that has been defrosted for longer than 48 h.
4. 2 % TCA in PBS. 10 % TCA (in H₂O) stock can be stored at −20 °C. Defrost and mix with 10× PBS and H₂O to make final concentration of 2 % in 1× PBS.
5. PBS: phosphate-buffered saline pH 7.3.

2.3 Brain Dissection Equipment

1. Glass petri dish.
2. Bunsen burner.
3. Oven (60 °C).
4. Minute pins A1.
5. Superfine Dumont forceps (Fine Science Tools).
6. Needle holders (Fine Science Tools or VWR)
7. Tungsten wire 0.125 mm.
8. 12v DC power supply.
9. Two wires with crocodile clips.
10. Glass jar with lid.
11. Electrode.
12. Microcentrifuge tubes.

2.4 Brain Dissection Reagents and Solutions

1. Sylgard: 1 kit 184 from Dow Corning WPI Sylgard <http://www.dowcorning.com/applications/search/products/Details.aspx?prod=01064291&type=&country=GBR>.
2. Saturated NaOH.
3. PBS.

2.5 Brain Dissection Sylgard Dishes

1. Sylgard is an elastomer curing agent. The Sylgard kit contains two solutions that set to a hard rubberlike texture when mixed. A layer of Sylgard in a glass petri dish makes a great dissecting dish as you can pin the embryos using insect/minute pins to the Sylgard, immobilizing them while you dissect. You also minimize damage to forceps and dissecting needles.
2. Mix Part A liquid from the Sylgard 184 kit with Part B liquid in a ration of 10:1 in a beaker. You will need 40–50 ml per petri dish.
3. Thoroughly mix the two solutions together using a disposable stirrer such as a tongue depressor. Be careful not to stir too vigorously so as not to create unwanted bubbles in the mixture.
4. After mixing, pour the Sylgard slowly into the glass petri dishes laid on a flat, non-vibrating surface where they can remain undisturbed for several days. Fill each petri dish to 1/3–1/2 full.
5. After the Sylgard has settled for a few minutes, you will see that bubbles have risen to the surface of the liquid. Burst these using a Bunsen burner by passing the flame lightly close to the surface of the Sylgard.
6. Put the petri dish lids on top to prevent dust settling on the plates while they set.
7. Sylgard dishes need at least 24 h to set and improve if left for several days. It is possible to speed up this process by heat curing them at 60 °C for several hours in a mini oven or incubator.

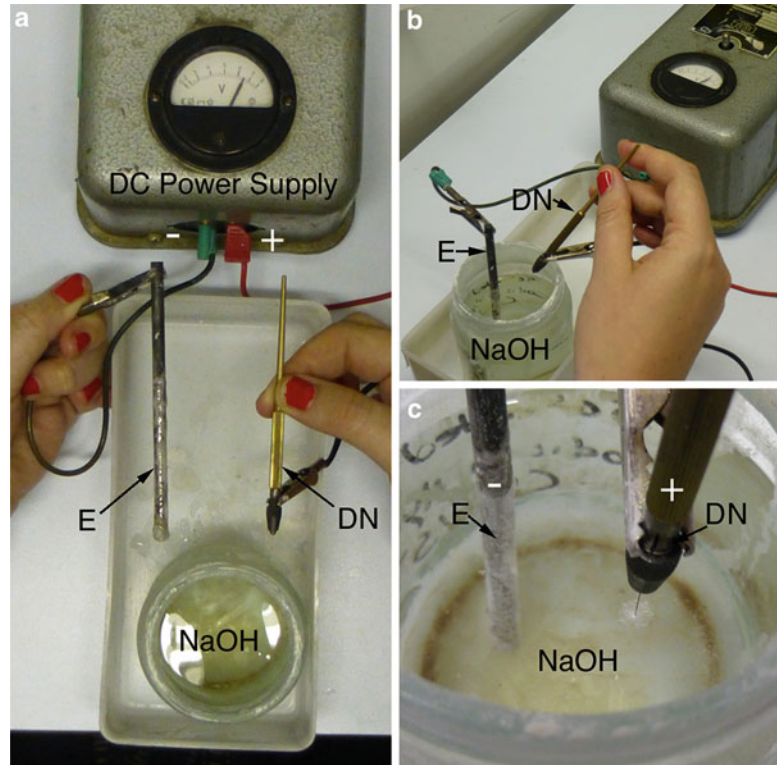


Fig. 1 Sharpening dissection needles using an electric current and saturated sodium hydroxide (NaOH). (a) Connect an electrode to the negative terminal of a 12v DC power supply. Connect your dissection needle to the positive terminal. (b) Submerge the negative electrode into the saturated NaOH. (c) Dip the tip of the tungsten wire into the NaOH and slowly draw back up out of the solution. Repeat this motion several times until you have a very sharp point. When making a new dissecting needle, you will have to repeat the dipping action several times. When you are sharpening a dissecting needle that has already been sharpened before, you only need to dip the needle two or three times

2.6 Brain Dissection Needles

To remove the skin and eyes from zebrafish embryos, we use dissecting needles fashioned from fine tungsten wire. The tungsten wire is clamped into a needle holder and cut using strong scissors so it protrudes from the tip of the holder by about 1 cm. The wire at this point is blunt but can be sharpened to a very fine sharp point using an electric current and saturated sodium hydroxide (Fig. 1).

2.7 Immunohistochemistry Equipment

1. 1.5 ml microcentrifuge tubes.
2. Plastic fine tip Pasteur pipettes or aspirator.
3. 50 ml Falcon tubes.

2.8 Immunohistochemistry Reagents and Solutions

1. PBS pH 7.3 + 0.5–0.8 % Triton-X100 (PBTr).
2. 50 % MeOH in PBS.
3. 100 % MeOH.
4. Proteinase K(PK) 10 mg/ml (this is a 1,000× stock) store in aliquots at –20 °C.
5. Trypsin (0.25 % in PBS). Trypsin stock is 2.5 % (10×).
6. Immunohistochemistry blocking solution (IB): For 1 ml of IB, 100µl normal goat serum (NGS), 10µl of DMSO, 0.89 ml PBTr.
7. Anti-GFP: polyclonal rabbit α -GFP from AMS Biotechnology gives great results. Use 1:1,000.
8. Anti-acetylated tubulin antibody: mouse monoclonal (IgG2b) from Sigma. Use 1:500 [11].
9. Anti-SV2: mouse monoclonal (IgG1) DSHB. Use 1:500 [12, 13].
10. Anti-GFP(Rat): rat monoclonal (IgG2a) from Nacalai Tesque. Use 1:1,000.
11. Chk pAb to GFP: chicken polyclonal from Abcam. Use 1:1,000 [15].
12. Anti-RFP: rabbit polyclonal from MBL. Use 1:2,500 [16].
13. Anti-DsRed (Living Colors): rabbit polyclonal from Clontech. Use 1:300 [14].
14. Anti-Kaede (M-125-3): mouse monoclonal from MBL. Use 1:200 (does not distinguish between red and green forms of protein).
15. Anti-Kaede (PM 102): rabbit polyclonal from MBL. Use 1:1,000 (does not distinguish between red and green forms of protein).
16. Secondary antibody depends upon primary antibody and detection method: For fluorescence, Molecular Probes (www.probes.com) Alexa Fluor 488 goat α -rabbit (highly cross-adsorbed) IgG. Use at 1:200. To detect anti-acetylated tubulin and anti-SV2 in the same sample, use isotype-specific secondaries: Alexa Fluor 568(IgG2b) and Alexa Fluor 633(IgG1). Use at 1:200. Molecular Probes also make fluorescent secondaries against rat and chicken primary antibodies conjugated with various fluorescent dyes.

2.9 Confocal Mounting and Imaging Equipment

1. Silicone grease: Fisher Scientific.
2. Glass rings for mounting.
3. Disposable Pasteur pipettes, glass, short tip.
4. Araldite Rapid: Fisher Scientific.
5. Rubber bulbs for pipettes 1 ml (VWR).

6. Small coverslips 22 × 22 (VWR).
7. Microscope slides (VWR).
8. Heat block set to 40 °C.
9. Microwave.
10. Beaker with microcentrifuge tube plastic stand to melt agarose.
11. Dissecting microscope.
12. Confocal microscope with appropriate laser lines.
13. Long working distance objective lens. Highest possible numerical aperture (NA). Approximately 500 µm (±250) working distance (WD) is required. For example, Leica HCX IRAPO L 25×/0.95 W is a water immersion lens with working distance of 2.4–2.5 mm, and coverglass and non-coverglass corrected versions are available.

2.10 Confocal Mounting and Imaging Reagents and Solutions

1. Fish water without methylene blue.
2. PBS.
3. Low gelling temperature agarose (Sigma): 1 ml aliquots of 1 % agarose in filter-sterilized fish water (with no methylene blue) or embryo medium (E3) for live time-lapse imaging. For fixed embryos use 1 % agarose in PBS or 1 % regular (not low melt) agarose in PBS/80 % glycerol. The latter solution can be challenging to make. Use a water bath and stirrer to make a 5 % agarose solution, and then add 4× volume of glycerol.
4. Tricaine:(3-amino benzoic acid ethyl ester) (Sigma) 400 mg tricaine powder, 97.9 ml DD H₂O, ~2.1 ml 1 M Tris (pH 9). Adjust pH to ~7. Freeze this solution into aliquots and use 4.2 ml per 100 ml of fish water or E3.
5. CyGEL Sustain (500 µl) (BioStatus Limited).
6. 60× E3 embryo medium: NaCl 3 M, KCl 0.1 M, CaCl 0.2 M, MgSO₄ 0.2 M dissolved in deionized water.
7. Ice pack or ice bucket.

2.11 Image Processing Software

1. Volocity (Perkin Elmer).
2. XuvTools.
3. ImageJ/Fiji.

3 Methods

3.1 Embryonic Culture and Fixation

1. Embryos should be collected and raised at 28.5 °C in fish water or embryo medium without methylene blue. This medium minimises the auto-fluorescence of the skin caused by

methylene blue that is not desirable with fluorescent imaging methods.

2. At 24 hpf transfer embryos to fish water containing PTU again without methylene blue. PTU inhibits pigmentation of the embryos.
3. Defrost sweet fix just prior to fixation at room temperature. Embryos should be dechorionated using forceps prior to fixation. Fix embryos in large volumes of sweet fix. Transfer embryos to 7 ml bijou tube; remove as much fish water as possible before adding sweet fix. Up to 200 embryos can be fixed per 5 ml of sweet fix (*see Note 1*).
4. Fix embryos at 25 °C (employ an incubator if necessary). 1 h of fixation per day of development, e.g., for 24 hpf embryos fix for 1 h at 25 °C.
5. After fixation, remove most but not all sweet fix and top up tube with PBS (do not use any detergent (e.g., triton) at this stage).
6. Leave embryos overnight at 4 °C before starting dissection. Embryos can be stored for several days at 4 °C before dissection but will deteriorate in that time so prompt dissection is advised. If the embryos are not going to be dissected prior to performing the immunohistochemistry, then dehydrate the embryos using these steps.
7. Transfer to 1.5 ml tubes, rinse 2×, and wash 3×10 min in PBTr (phosphate-buffered saline + 0.5–0.8 % Triton-X100) on side on shaker.
8. Transfer to MeOH—1×5 min wash in 50 % MeOH/50 % PBTr and 3×5 min in 100 % MeOH. NB Mixing PBTr and MeOH is exothermic—this solution must be made up at least 15 min before use.
9. Store at –20 °C for at least 6 h (or up to 6 months+) to delipidate.
10. For most antibodies formaldehyde fixation with sweet fix gives the best results; however, some antibodies work better with TCA fixation. This is often true of older embryos (5dpf+). For TCA fixation, simply replace 2 % TCA for sweet fix in the protocol above. TCA-fixed embryos should be permeabilized using trypsin (*see below*); TCA-fixed embryos are also more opaque and will benefit from clearing in glycerol at the end of the protocol (*see below*).

3.2 Brain Dissection

Dissecting the skin and eyes off of zebrafish embryos prior to antibody staining vastly improves the penetration of antibodies and reduces auto-fluorescence of the skin common to many transgenic lines. Although this technique requires some dexterity, with practice it becomes routine and the results justify the effort (Fig. 2).

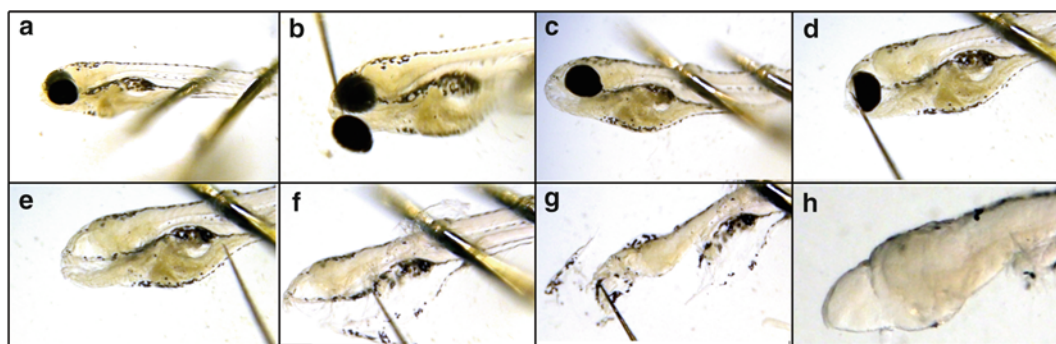


Fig. 2 Dissection. (a) Pin embryo laterally to a Sylgard dish. (b) Remove eye using a sharpened tungsten needle. (c) Remove pins and flip the embryo so the other side now faces upwards. (d) Remove second eye using sharpened tungsten needle. (e) Cut away the yolk and jaw from the ventral surface of the embryo. (f) At the level of the otic vesicle using either a sharpened tungsten needle or fine forceps, grip a piece of skin and tease away from the brain. (g) Pull this flap of skin rostrally, removing all of the skin from the surface of the brain. (h) Close up showing completely dissected brain ready to be transferred into methanol for immunohistochemistry or in situ hybridization

1. Under a dissecting microscope, transfer embryo to Sylgard dish.
2. Using forceps (a low-grade type), pin the embryo with minute pins. Embryo should be pinned on its side (laterally). Pin the embryo twice through the tail at the level of the notochord. Push down on the pins so they stick into the Sylgard.
3. Using a dissecting needle, gently cut the skin surrounding the eye and tease the eye off the head. The eye should pop off easily at stages post-48 hpf but is trickier to remove at earlier stages.
4. Unpin embryo, taking care not to break the tail. Turn the embryo to lie on the opposite side and re-pin to the Sylgard.
5. Remove second eye using the same procedure as above.
6. Cut skin between anterior yolk and heart/head using dissecting needle. Then cut away the skin attaching the yolk sack to the body. The yolk should come off as an intact structure.
7. Remove the jaw and other connective tissue from the ventral surface of the brain.
8. Make a shallow cut at the level of the otic vesicle. Using fine forceps tease a flap of skin off and pull this flap rostrally removing all of the skin from the surface of the brain.
9. Using fine forceps (*see Note 2*) or a dissecting needle, pull off any remaining skin, leaving just the brain attached to the tail.
10. Unpin the embryo and transfer to a 1.5 ml tube filled with PBTr using normal forceps.

11. After all embryos are dissected and transferred to 1.5 ml tube. Wash several times with PBTr, then transfer to MeOH (*see* Subheading 3.1), and store at -20°C until you want to start the immunohistochemistry (*see* **Note 3**).

3.3 Fluorescent Immunohistochemistry Protocol

1. Rehydrate embryos: Wash embryos 5 min in 50 % MeOH/50 % PBTr, and wash 3×5 min in PBTr.
2. Permeabilize embryos (PFA-fixed embryos): Proteinase K (pK) digestion times vary with embryo age, and also pK batches vary. The times below are a guide, and tests should be carried out with the particular batch employed (store pK at -20°C at 10 mg/ml—this is 1,000 \times stock). Dilute pK stock 1/1,000 (1 \times) in PBTr.
 - Up to tailbud, no PK
 - 2–10ss, in and out of 1 \times PK
 - 10–15ss, 1 min 1 \times PK
 - 16–26ss, 2 min 1 \times PK
 - 24 h, 10–15 min 1 \times PK
 - 30 h, 20 min 1 \times PK
 - 36–48 h, 30–40 min 1 \times PK
 - 2.5d, 30–40 min 1.5 \times PK
 - 3d, 30–40 min 2 \times PK
 - 4d, 30–40 min 3 \times PK
 - 5d, 30–40 min 4 \times PK.

Digestions are at room temperature (18–22 $^{\circ}\text{C}$) with tube lying on its side. Dissected embryos should be treated as somite-stage embryos (minimal permeabilization). Rinse 3 \times in PBTr. Postfix in 4 % PFA for 20 min at room temperature (also denatures pK). Wash 3×5 min in PBTr.

3. For TCA-fixed embryos: Rinse embryos 3×5 min in PBS. Prechill trypsin solution (0.25 % in PBS) and 5 ml per sample of PBTr on ice until cold. Incubate embryos in trypsin, on ice for 5–10 min (according to age 36 h to 5 days), longer for older embryos, depending on trypsin batch; titrate upon first use. Rinse 2 \times in cold PBTr then 3×10 min in cold PBTr, bring to RT.
4. Block endogenous binding sites: Incubate in IB for at least 1 h at room temperature on shaker.
5. Primary antibody incubation: Incubate in IB + primary antibody overnight at 4 $^{\circ}\text{C}$ on shaker. Some antibodies work better with longer incubations or room temperature incubations, and for room temperature incubation, consider adding 2 mM sodium azide to the IB to inhibit mold growth. For longer incubations increase the number and length of post-incubation washes.

6. Postprimary incubation washes: Remove primary antibody (can be kept at 4 °C for reuse within a week). Rinse 3× in PBTr. Wash at least 4×30 min in PBTr on shaker, can be longer/more washes.
7. Secondary antibody incubation: Incubate in IB + secondary antibody overnight on shaker at 4 °C or room temperature.
8. Postsecondary incubation washes: Rinse 3× in PBTr. Wash at least 4×30 min on shaker. Fluorescent-stained embryos are now ready to be imaged. Transfer either to 80 % glycerol (through 25 % and 50 % glycerol/PBS solutions) and mount. Alternatively, keep in PBTr and mount in agarose for imaging. Keep at 4 °C in the dark and image as soon as possible.

3.4 Antibodies for Neuroanatomy

Zebrafishbrain.org focuses mainly on the characterization of transgenic zebrafish lines. Many hundreds of transgenic and enhancer trap lines have been created by the zebrafish community and can be generated easily using protocols provided elsewhere. To generate data for zebrafishbrain.org, high-resolution confocal

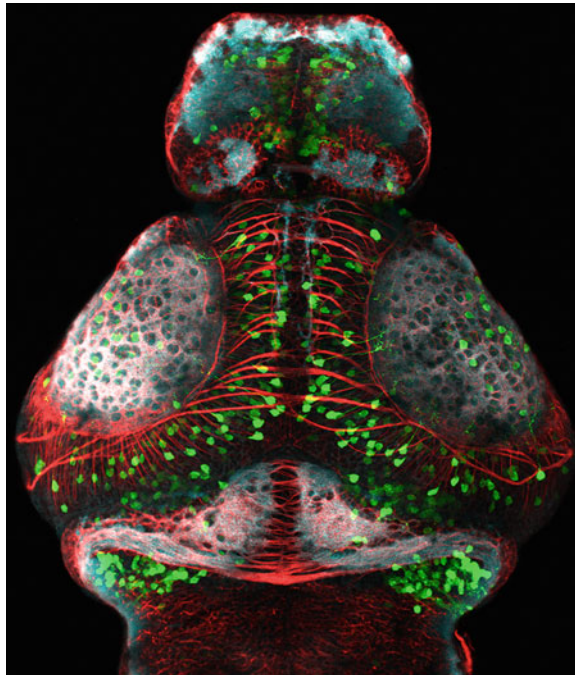


Fig. 3 Transgenic zebrafish embryo labelled with anti-GFP (*green*), anti-SV2 (*blue*), and anti-acetylated tubulin (*red*). This is a dorsal view of a 4dpf Tg(dlx 4/6:GFP) embryo. In this image we can see GFP-positive cells in the telencephalon, optic tectum, and the cerebellum. The SV2 staining labels the synaptic neuropil and axonal tracts are visualized by labeling with anti-acetylated tubulin antibody. This is an example of the type of images we use to populate the zebrafishbrain.org database (Image generated by Monica Folgueira)

imaging is performed on suitable transgenics generated in-house and externally that have fluorescent expression in specific brain regions or nuclei (Fig. 3). With the majority of transgenic lines expressing GFP, the primary antibody used most frequently is a polyclonal rabbit anti-GFP from AMS Biotechnology (TP401). This antibody has excellent penetration and works equally well in whole-mount embryos at all stages and on sections.

To aid anatomical orientation transgenic specimens can also be labelled with anti-acetylated tubulin (IgG2b, Sigma) and/or anti-SV2 (IgG1, DSHB). These antibodies label beautifully the axonal connections and neuropil, respectively. In addition to these antibodies being informative from a neuroanatomical perspective, they are also invaluable as a tool for anatomical localization. These antibodies can be used as a framework to easily compare the expression patterns of different transgenic lines and locate GFP-positive structure in the context of the brain. Both antibodies are mouse monoclonals; fortunately, they are different subtypes and can be detected in the same specimen using subtype-specific secondary antibodies.

We have trialed many neurotransmitter antibodies in whole-mount zebrafish embryonic/larval preps with little success. Colocalization to check which neurotransmitters a particular cell is expressing in a transgenic normally needs to be done using immunohistochemistry on cryosections. There is a protocol for this on zebrafishbrain.org. Some exceptions of antibodies that have worked well in whole-mount can also be seen there. Different fixation methods can improve the efficacy of some of these antibodies for immunohistochemistry. Many antibodies that do not work after PFA fixation will work better after fixation with TCA or other fixatives such as glutaraldehyde.

3.5 Cell Dyes for Neuroanatomy

Using a nuclear label in conjunction with a fluorescent immunostaining can be very useful to delineate brain nuclei, neuropil, and ventricles through the tissue (Fig. 4). Nuclear staining has also been employed by the Driever lab to produce a 3D reference brain for their ViBE-Z software. They have also used acetylated tubulin immunohistochemistry with their reference brain [17, 18].

For nuclear staining, use SYTOX Orange or Green at a concentration of 1:10,000 or Topro3 at a concentration of 1:5,000 depending upon the wavelengths required. These dyes can be added with the secondary antibody incubation. Staining with nuclear dyes works best following room temperature incubation in the dye so it is advisable to add 2 mM azide with the secondary antibody/nuclear dye IB mixture to inhibit mold formation. The dyes bleach very easily so minimize exposure to light and keep the laser intensity on the confocal as low as possible when imaging, and also, timely imaging following the staining process produces the best results.

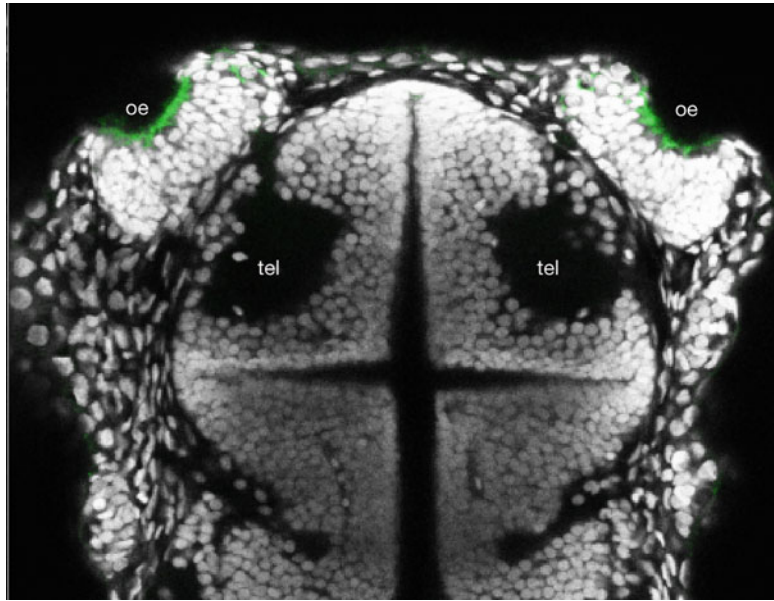


Fig. 4 Nuclear dyes. Nuclear dyes show brain nuclei and also expose morphological boundaries between brain areas. Neuropil is not marked and is revealed as dark spaces. In this confocal slice through the forebrain of this enhancer trap line, we can see expression of GFP in the olfactory epithelia (Image generated by Tim Geach and TAH)

3.6 Confocal Mounting with Glass Ring

1. Mounting media: The choice of mounting medium depends upon the experimental procedure that is being undertaken. For live imaging (e.g., timelapse), embryos can be mounted in agarose (made with fish water) or CyGEL Sustain (*see* Subheading 3.9 below). For imaging of fixed preparations, embryos can be mounted in agarose (made with PBS) or glycerol agarose. Agarose in simple PBS leaves embryos slightly opaque; depth penetration can be improved if the embryo is mounted in glycerol agarose (as described in the protocol associated with the recent Driever lab paper: [17, 18]). The methods of ring mounting described below can be achieved using either aqueous or glycerol agarose. Alternatively, embryos can be mounted between stacked coverslips. Aqueous agarose is slightly preferable as a mountant when using water immersion lenses, and glycerol immersion lenses should be employed where available when mounting in glycerol agarose. Water immersion lenses can produce acceptable results with glycerol agarose as a mountant despite being an optically suboptimal system.
2. Mounting in glass rings (Fig. 5): The advantage of this preparation is that the embryos are securely mounted in a large volume of agarose that permits the orientation of the embryos as is required for imaging. It can be achieved using either aque-

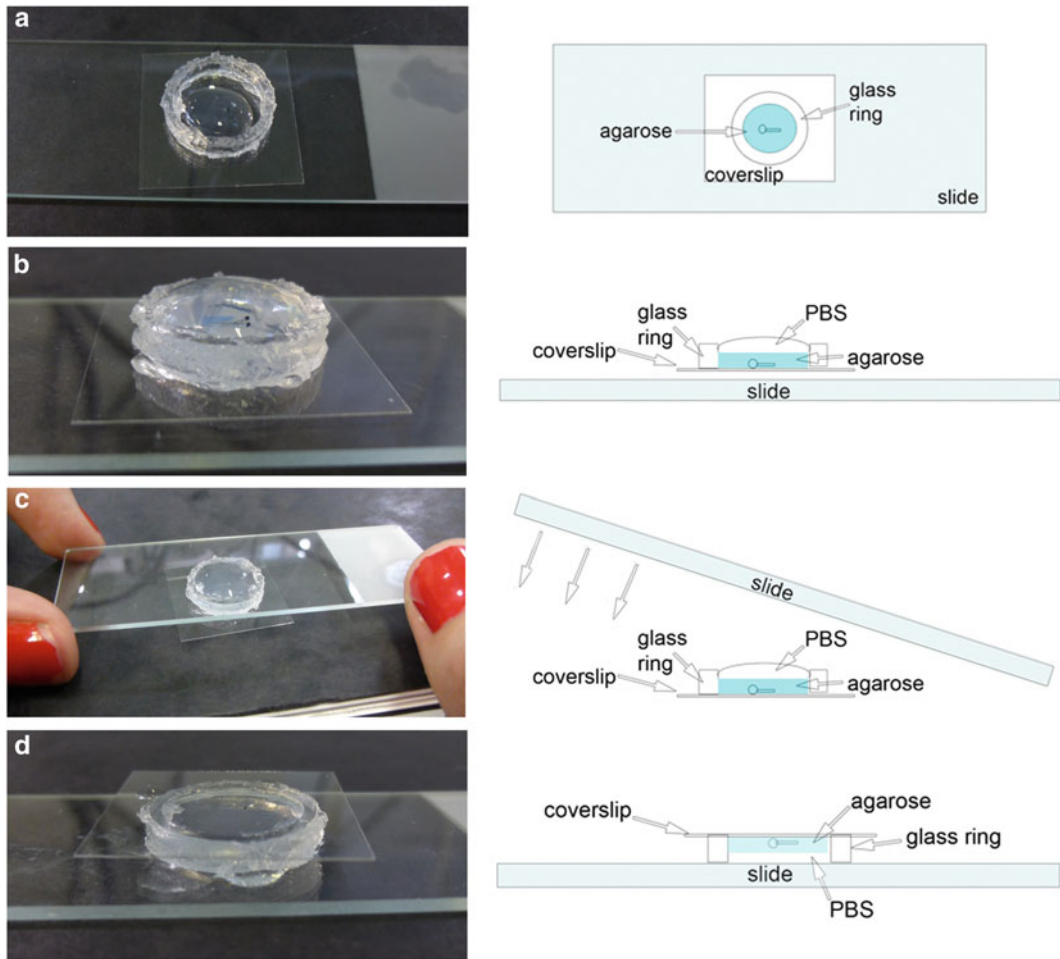


Fig. 5 Mounting in glass rings. **(a)** On a dissecting microscope, place a 22×22 coverslip onto a slide. Smear silicone grease on both sides of a glass ring, and press it onto the coverslip forming a watertight seal. Pipette the embryo into the agarose and then onto the coverslip in the center of the glass ring. Orientate the embryo so the surface of the embryo you wish to image is against the coverslip. **(b)** Fill the ring 2/3 full with agarose and let it set. Once the agarose has set firm, pipette enough PBS onto the surface of the agarose to form a convex meniscus over the top of the ring. Overfilling the ring with PBS means that no bubbles will be trapped when you place the slide on top. **(c)** Take another slide and press down onto the top of the glass ring expelling the excess PBS. Make sure the slide and the glass ring have formed a watertight seal with the silicone grease. **(d)** The prep can now be inverted with the coverslip on top and slide on the bottom. Your embryo will now be at the top of the prep just under the coverslip ready for imaging

ous or glycerol agarose as the mounting medium although glycerol agarose should only be used for fixed preparations. Before beginning this protocol, several tubes (as many as required) of agarose should be melted in the microwave and transferred to a heat block set at 45°C .

3. Place a slide on the dissecting microscope.
4. Place a 22×22 coverslip onto the slide.

5. Smear silicone grease on to the top and bottom of a glass ring, and press it onto the coverslip to form a watertight seal.
6. Using a glass pipette, suck up the embryo to be mounted in a small volume of medium, and pipette it into the molten agarose, which should be around 40 °C (slightly cooled from the hot block temperature). Refill the pipette with 0.5–1 ml of agarose with the embryo. Expel the pipette contents (agarose and embryo) onto the coverslip in the center of the glass ring. There should be enough agarose to fill the glass ring around two-thirds full.
7. Moving quickly, using forceps or other suitable tool, manipulate the embryo to place in the correct orientation for imaging. Bear in mind that after **step 7**, the preparation will be inverted; thus the side of the embryo to be imaged should be closest to the coverslip; for example, if the embryo is to be imaged from a dorsal aspect, the dorsal side of the embryo will be touching the coverslip with the ventral side facing upwards at this point.
8. Once the agarose has set firm, pipette some PBS (*see Note 5*) onto the surface of the agarose to form a convex meniscus over the top of the ring (it is important to slightly overfill the ring as this prevents bubbles getting trapped inside the ring once the top slide is secured).
9. Take another slide and press down onto the top of the glass ring starting from a slight angle, expelling the excess PBS. Make sure the slide and the glass ring have formed a watertight seal with the silicone grease. The prep can now be inverted with the coverslip on top and slide on the bottom. Use a tissue to remove the excess liquid. Your embryo will now be at the top of the prep just under the coverslip, ready for imaging.

3.7 Confocal Mounting Without Glass Ring

If glass rings are not available, a similar mounting method can be used where a well of silicone grease is constructed directly onto a slide (Fig. 6). A syringe filled with silicone grease should be used to squeeze the grease onto the slide. The embryo is mounted on a coverslip as above in a large drop of agarose (the diameter and depth of this drop should not exceed the width and depth of the silicone grease well). The silicone grease well is filled with PBS to form a convex meniscus. The coverslip with the embryo attached is inverted and pressed gently down into the well expelling the excess PBS. Make sure the coverslip forms a watertight seal with the top of the silicone grease well. The embryo is now ready for imaging. This method also has the advantage of allowing for a small adjustment in the orientation of the sample.

3.8 Mounting of Live Embryos for Confocal Imaging

For imaging of live embryos (particularly for timelapse), embryos can be mounted in a large epoxy resin chamber or well. This allows for constant gaseous exchange and thus improves the health of the embryo. These chambers are easily made using Araldite or similar

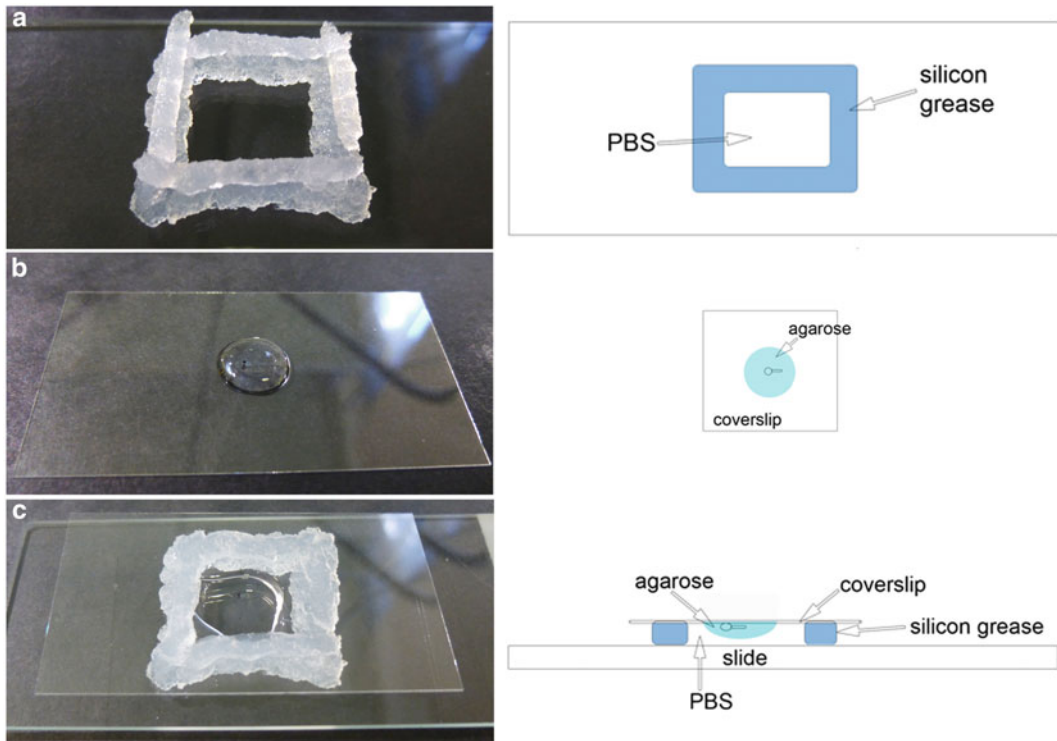


Fig. 6 Mounting fixed embryos in silicone grease wells. (a) Using a syringe filled with silicone grease, pipe a well onto a microscope slide. (b) On a coverslip orientate your embryo in a drop of 1 % agarose. The surface of the embryo you wish to image should be touching the coverslip. Half fill the silicone grease well with PBS. (c) Once the agarose is set, invert your coverslip so that the embryo is submerged in PBS inside the silicone grease well. Lightly push down on the coverslip to create a sealed chamber

epoxy compounds. Mix the two components and make a rectangular well, a few millimeters deep on a clean slide. Make sure the well is continuous so the liquid won't leak out. Allow the resin to polymerize until hard before using. These large wells mean that it is possible to mount several embryos on the same slide. This is particularly useful for multiple timelapses on a confocal with a motorized X/Y stage. Once made, these slides can also be reused many times. Mounting protocol:

1. Anesthetize embryo(s) in a petri dish by adding tricaine (final concentration 1.6 mg/l) to the fish water. Wait until the embryo has completely stopped twitching before trying to mount. Check that heart beat of the embryo is still strong under the dissecting scope.
2. Place resin chamber slide onto the dissecting scope.
3. Remove tube of agarose from the heat block and allow to cool down to below 37 °C to avoid heat-shocking the embryo.
4. Pipette the anesthetized embryo into the warm molten agarose then refill the pipette with agarose and the embryo. Pipette

embryo and agarose onto the slide with enough agarose to form a small bubble around the embryo.

5. Orientate the embryo very gently using forceps or other appropriate implement. This prep will not be inverted so the side of the embryo to be imaged should face upmost; it should also not be too deep in the agarose.
6. Repeat this procedure for two or three other embryos per slide. Do this as quickly as possible so the embryos mounted earlier do not dry out.
7. When the agarose is set, flood the chamber with fish water containing tricaine.
8. The embryos are now ready for imaging. Embryos mounted in this way should be imaged using a non-coverslip corrected water immersion lens. These lenses can be dipped straight into the fish water as long as it does not contain methylene blue. For the modifications required to adapt this method to live embryos (*see Note 4*).

3.9 Mounting of Live Embryos Using CyGEL Sustain

An alternative to agarose for mounting live embryos is a compound called CyGEL Sustain. CyGEL is liquid at low temperatures and changes from a sol to a gel at 23–24 °C. The sol–gel conversion can be reversed, by simply placing the sample on ice for a few seconds. This is particularly useful for short procedures that require a fast and easy way of recovering the embryo undamaged after manipulation, such as electroporation or Kaede photoconversion. CyGEL Sustain is a compound commercialized by BioStatus Limited (www.biostatus.com). They provide protocols on their web page, but these are optimized for the growth of cells. Here we provide a protocol adapted for zebrafish mounting:

1. Place vial of CyGEL Sustain on ice and make sure it is a sol.
2. Add E3 so that the final concentration will be 1× (8.4 µl of 60XE3 for 500 µl of CyGEL). Keep it on ice.
3. Prepare a chamber on a slide with silicone grease, or use a glass ring as described in Subheading 3.6.
4. Warm the vial slightly using hands so that it is not too cold (but not too much because it gels).
5. Pipette the anesthetized embryo into the CyGEL then refill the pipette with the embryo and enough CyGEL to fill the silicone chamber or glass ring.
6. Orientate embryo as required the consistency of CyGEL quickly becomes dense, becoming a tight gel within minutes. At that point, the embryo is ready for microscopy.

No additional liquid should be placed on the gel as it immediately becomes a sol again. If the embryo needs to be imaged using

a water immersion lens for a long period, it should be mounted upside down in a glass ring on a coverslip filled completely with CyGEL and tightly sealed with a slide. This ensures that the preparation stays moist for the length of the procedure and that no extra liquid disrupts the gel. Tricaine should be added to the E3 medium (4.2 ml tricaine per 100 ml E3) used to prepare the CyGEL, to ensure that the embryo remains anesthetized for the length of the procedure.

3.10 Confocal Microscopy Setup

Setup of the imaging system for optimal imaging depends very much on the individual sample employed. High-NA, long working distance objectives (water and/or glycerol immersion) are available from most microscope manufacturers. These lenses are ideally suited to imaging the zebrafish brain as they permit imaging through the whole depth of the brain at high resolution. There are many tradeoffs in the setup of the imaging parameters. For high-quality three-dimensional reconstructions when imaging fixed, antibody-stained preparations, it is best to aim for approximately isometric voxels at resolutions approaching the limit of diffraction; however, this should be traded off against the time taken and the file size. For live imaging of fluorophores, it is best to minimize bleaching of the (usually dim) fluorescent proteins. Thus minimizing the dwell time at any particular voxel is advisable.

3.11 Image Processing

To process/visualize images, there are a number of free and commercial packages available. To produce zebrafishbrain.org, we mainly use Volocity (PerkinElmer), but other software or a combination of packages can be used for processing/visualization. Examples of other packages are Imaris (Bitplane), Amira, FluoRender, OsiriX, Drishti, and ImageJ/Fiji. There are also some web-based processing servers that are available to do various tasks (e.g. XuvTools and ViBE-Z).

4 Notes

1. Depending on the antigen certain antibodies may work better after fixation with TCA: 2 % TCA in PBS for exactly 3 h at RT in 5 ml bijoux. Transfer to 1.5 ml tubes, rinse 2×, and wash 3×5 min PBS (on side). Store at 4 °C for a week, but add 20 mM azide if storing for longer (to prevent mold). In azide PBS embryos should keep for a month.
2. Take great care of superfine grade forceps. Do not use them to touch anything except the embryo, and try not the stick them into the Sylgard as even this can bend and blunt them. To pin and manipulate the embryo, use normal dechoriation-grade forceps. Superfine forceps are expensive and not strictly neces-

sary for dissecting. A combination of dissecting needle and normal forceps can be sufficient. Forceps can be rehoned using fine pliers and a whetstone.

3. It is possible to dissect an embryo without turning it half-way through. See http://zebrafishbrain.org/movies/brain_dissection.
4. To mount live embryos in glass rings for timelapse. Follow the procedure described in Subheading 3.4.2 with the following changes. Embryos must first be anesthetized by adding tricaine to the fish water prior to mounting. Use 0.5–0.8 % low melt agarose in filtered fish water or E3. Use fish water with tricaine instead of PBS to flood the ring.
5. Glycerol agarose sets very slowly. When using glycerol agarose, replace the PBS used to top up the ring with 80 % glycerol/PBS.

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