

Versatile Dual Mounting Enables Larval Zebrafish Imaging Across Microscope Configurations

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Abstract

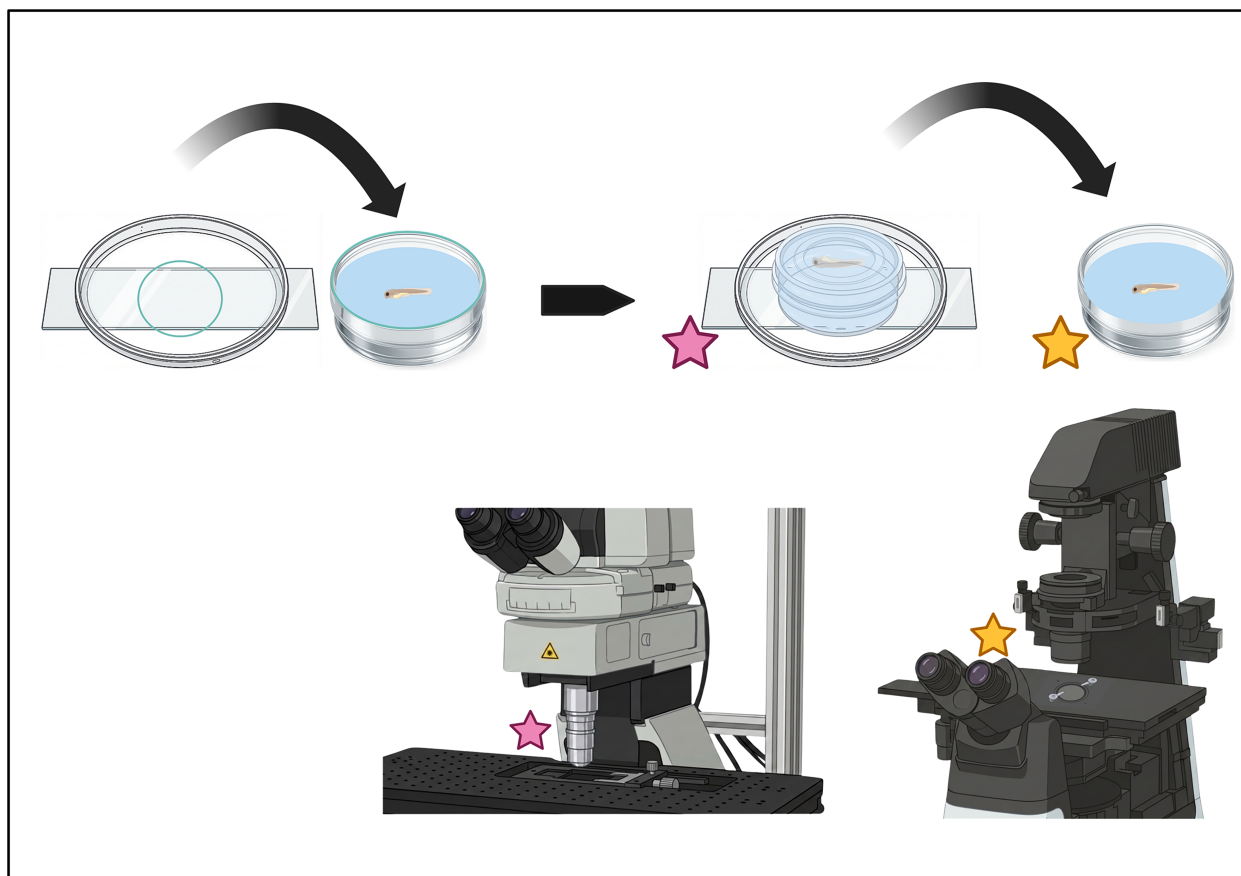
Larval zebrafish are often mounted laterally to ensure consistent anatomical positioning and to standardize imaging of body axes across early development. However, this conventional approach often tethers sample orientation to a single microscope configuration and limits optical accessibility. We present a mounting protocol for larval zebrafish that enables optical access from both dorsal and ventral orientations while preserving lateral sample position. This approach uses common laboratory consumables to establish a mounting platform that eliminates any need to remount samples between the use of upright and inverted microscopes. By establishing a hydrophobic seal, mounted embryos can be inverted with ease to access the sample from either orientation. A seamless transition here facilitates reliable identification and longitudinal tracking of the same biological region of interest across microscope configurations. This protocol is broadly applicable to live imaging experiments requiring flexibility in imaging geometry, minimal sample handling, and high reproducibility.

Key features

- Dual-configuration mounting setup can be established from standard, low-cost consumables in any laboratory setting.
- Preserves lateral mounting in agarose for consistent anatomical positioning and experimental continuity.
- Enables seamless transition between upright and inverted microscopes without the need for specimen remounting.

Keywords: Zebrafish, Larvae, Mounting, Inverted, Upright, Microscopy

Graphical overview



Dual-mounting configuration to transition between upright and inverted microscopes

Background

Zebrafish (*Danio rerio*) have become a widely popular vertebrate model organism given their early larval transparency, highly conserved genome with humans, external embryo fertilization, and rapid development compared to other vertebrates. These features position zebrafish as a well-suited model for *in vivo* observations across fields like developmental biology, neuroscience, genetics, and toxicology [1,2]. This has enabled their use as a robust predictive model for teratogenicity, with morphometric profiling of development offering insights into chemical-specific consequences and modes of action conserved across vertebrates [3]. More broadly, many modern experimental paradigms integrate different imaging modalities with genetic manipulations and behavioral assays to both influence and capture biological dynamics with larval zebrafish in real time [4]. However, fully exploiting these capabilities with non-invasive imaging techniques often depends on mounting orientation and optical accessibility.

These practical constraints become particularly important when selecting optical imaging modalities that differ in penetration depth, speed, and configuration. Techniques like calcium imaging, photochemical conversion of fluorophores via targeted stimulation, optogenetics, and photoinduced ablation benefit from the advantages of multiphoton microscopy, including deeper penetration than in linear techniques like confocal imaging [5]. A great example involves the increased engineering of *in vivo* indicators to exploit the nonlinear physics of multiphoton excitation. Recent advancements have leveraged rhodopsin and calcium-based sensors to establish genetically encoded calcium (GECI) and voltage (GEVI) indicators across wavelengths that maximize photon capture and signal-to-noise ratio in deep tissue [6,7]. On the other hand, the faster imaging speeds, greater ease of setup, and robust optical sectioning provided by linear microscopes, specifically confocal systems, are especially useful when imaging rapid biological dynamics where temporal resolution and precision in optical sectioning are required [8]. Combining both approaches can integrate the depth of multiphoton penetration while maintaining the rapid visualization and temporal resolution provided by confocal imaging, but the ease of use with larval

embryos can be limited by opposing microscope objective orientations. Though varying modalities can be integrated into a single system, like the addition of a photostimulation laser to a spinning-disk confocal system, doing so often entails substantial cost and complexity.

Traditional lateral mounting for inverted imaging positions larval zebrafish against the bottom of a glass-bottom imaging dish, secured with a mound of agarose. This configuration allows for clear visibility of anterior–posterior axis development in zebrafish and helps to standardize anatomical positioning across samples. However, because the imaging dish is filled with liquid and embryos are fixed against the bottom of the dish, optimal accessibility is limited to imaging through the glass from below. Observation from above, as with top-down microscopes, is constrained by working distance limitations and obstruction from the mounting agar and anesthetic solution. This restricts flexibility across microscope geometries, as samples can only be imaged with the shortest optical distance from one side. Additionally, direct water immersion with the upright objective into the imaging dish risks damage to samples and mounting. The optical distance can be extended to target the region of interest across configurations without remounting, but this increases the limitations of multiphoton imaging in photobleaching, light scattering, and heat exposure [8]. Further, remounting samples can introduce variability in anatomical positioning, increase the required handling of anesthetized samples, and complicate the identification of the same biological region of interest for longitudinal assessment.

Here, we present a dual-configuration mounting model that enables easy transition across upright and inverted microscope geometries. This setup combines standard zebrafish mounting with a model constructed from common low-cost laboratory consumables to eliminate remounting across microscope configurations, supporting both upright and inverted imaging. Using a simple petroleum jelly assembly, we combine a 35 mm glass-bottom confocal imaging dish with a 60 mm Petri dish glued onto a microscope slide. This method successfully preserves the conventional lateral embryo positioning while enabling optical access from both upright and inverted perspectives to allow for rapid transition between imaging geometries. Ultimately, this configuration supports workflows that combine targeted interventions with long-term imaging and can be applied using any mounting orientation.

Materials and reagents

Biological materials

1. *Tg(erbb3b:gal4)^{nkgxai:GFFD37A}*, *Tg(UAS:eGFP)^{nkuasgfp1a}*, and *Tg(olig2:dsRed2)^{vu19}* zebrafish transgenic lines were used for mounting protocol validation [9,10].

Reagents

1. Instant Ocean salt (Instant Ocean, catalog number: 77780); store in an airtight container in a cool, dry place at room temperature (RT)
2. 3-Aminobenzoic acid ester (tricaine) (Pentair, catalog number: TRS1); store in an airtight container in a cool, dry place at RT
3. Tris (Tris[hydroxymethyl]aminomethane or Trimethamine) (Bio-Rad, catalog number: 1610716); store in an airtight container at RT
4. 1-Phenyl-2-thiourea (PTU) (Sigma, catalog number: P7629, CAS number: 103-85-5); store in an airtight container at RT, protected from light
5. Agarose, low gelling temperature (Sigma, catalog number: A94114); store in an airtight container at RT

Solutions

1. Egg water (see Recipes)
2. PTU stock solution (10×) (see Recipes)
3. PTU water (see Recipes)
4. Buffered tricaine methanesulfonate (MS-222) stock solution (see Recipes)
5. Anesthetic solution (see Recipes)
6. 0.8% low-gelling agarose (see Recipes)

Recipes

1. Egg water

Reagent	Final concentration	Quantity or volume
Instant Ocean salt	300 mg/L	300 mg
Deionized H ₂ O	–	1 L

2. PTU stock solution (10×)

Reagent	Final concentration	Quantity or volume
PTU	0.03%	300 mg
Deionized H ₂ O	–	1 L

3. PTU water

Reagent	Final concentration	Quantity or volume
PTU 10× stock solution	0.003%	100 mL
Egg water	–	900 mL

4. MS-222 stock solution

Reagent	Final concentration	Quantity or volume
Tricaine	4 g/L	4 g
Deionized H ₂ O	–	up to 1 L
1 M Tris (pH 9.0)	21 mM	21 mL

5. Anesthetic solution

Reagent	Final concentration	Quantity or volume
MS-222 stock solution	4%	400 µL
PTU water	0.003%	10 mL

6. 0.8% low-gelling agarose

Reagent	Final concentration	Quantity or volume
Low-gelling agarose	0.8%	8 mg
Egg water	–	1 mL

Laboratory supplies

- Breeding tanks (Aquaneering, catalog number: ZHCT100)
- Dumont Tweezers, pattern #5; 0.10 × 0.06 mm tip size (Roboz Surgical, catalog number: RS-5065)
- 100 × 15 mm Petri dish (Falcon, catalog number: 351029)
- 35 mm glass-bottom confocal dish, non-treated (Avantor, catalog number: 75856-746)
- 60 mm Petri dish (Fisher, catalog number: FB0875713)
- Disposable glass Pasteur pipettes (VWR, catalog number: 53283-916)
- Pipette pump, 10 mL (Honbay, ASIN: B07P61YDXB)
- Micro dissecting needle holder, 4 3/4" (Roboz Surgical, catalog number: RS-6061)
- Insect pins 0.30 mm size 00 (Roboz Surgical, catalog number: RS-6081-30)
- Glass microscope slides (Sail Brand, catalog number: 7105)
- 50 mL borosilicate glass beaker (Maccx, catalog number: BKL050-012)
- Borosilicate glass test tubes with autoclavable caps (ULAB, catalog number: UTT1009)
- Petroleum jelly (Vaseline, catalog number: UNI34500)
- Super glue (Krazy Glue, catalog number: KG58548R)
- Wood applicator stick, 6" × 1/12" (Oxford Instruments, catalog number: ID 51-1625-0580)
- Zeiss immersion W oil 2010 (Microscope World, catalog number: 444969-0000-000)

Equipment

1. Fisherbrand Basic 180 L Gravity Incubator (ThermoScientific)
2. ECLIPSE Ti2 (Nikon) equipped with a Dragonfly 200 high-speed confocal platform (Andor Oxford Instruments), a 40×/1.15 W APO LWD numerical aperture water immersion objective (Nikon), and a motorized stage (Applied Science Information)
3. Air-MP (Nikon), equipped with a 25×/1.10 W numerical aperture water immersion objective (Nikon), ProScan III motorized stage (Prior Scientific), Chameleon Vision II laser system (Coherent), and an HEC series Thermo-con cooling system (SMC)

Software and datasets

1. Fusion 2.6.0 (Andor Technology; 2026, license required)
2. Nikon Imaging Software, Elements Advanced Research 5.21.03 (Nikon; 2020, license required)
3. Imaris 11.0 (released 2025; license required)

Procedure

A. Obtain zebrafish embryos

Note: This section details the process of gathering embryos for use in microscopy experiments.

1. In the afternoon, prepare breeding tanks with a desired female-to-male ratio of 2:2.
2. The following morning, collect and store the freshly laid embryos in a Petri dish filled with egg water.
3. Rear embryos in a 28.7 °C incubator, replacing egg water with PTU at 24 h post-fertilization (hpf) to prevent the formation of pigmentation.
4. Manually dechorionate embryos with fine tip tweezers as needed by 48 hpf to preserve trunk linearity.
5. Keep embryos at 28.7 °C until the desired imaging time point, to ensure proper developmental pace [11].

B. Create mounting model

Note: This section outlines the creation of the dual-configuration mounting model for use across orientations with traditional mounting.

1. For compatibility with a standard microscope stage, use super glue to adhere the edge of a 60 mm Petri dish lid to a glass microscope slide (Figure 1). Let it cure for at least 10 min before use.

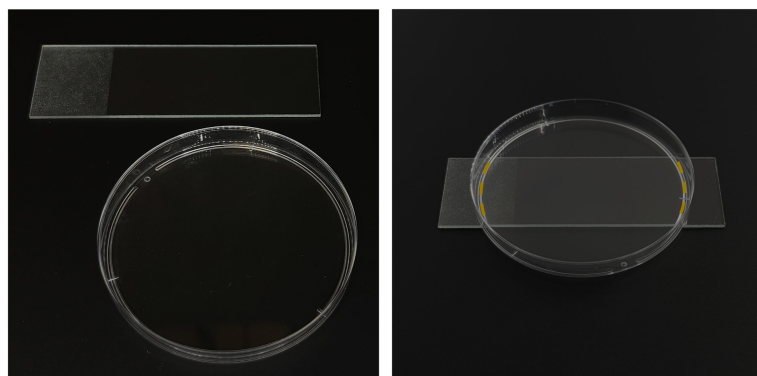


Figure 1. Assembly of the 60 mm Petri dish lid and glass slide (left) with super glue application along the dashed yellow lines (right)

Note: Modeling clay can be used as an alternative adaptation, ensuring that the Petri dish lies both as flat and as close to the glass slide as possible (Figure 2). However, if the microscope has an adjustable stage holder, the use of a glass slide is not necessary, and the 60 mm Petri dish lid can be directly accommodated.

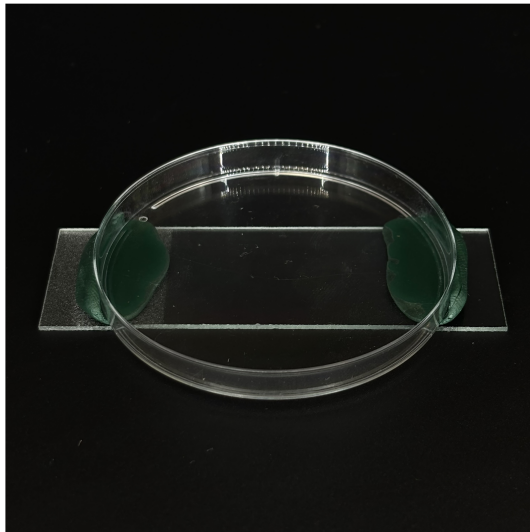


Figure 2. Alternative adherence of 60 mm Petri lid edges to a glass slide using modeling clay

2. Either by hand or with a wood applicator stick, line the rim of a 35 mm glass-bottom imaging dish to stamp a circle onto the 60 mm Petri dish lid (Figure 3). This will serve as a placement guide and reinforce the intended hydrophobic seal.

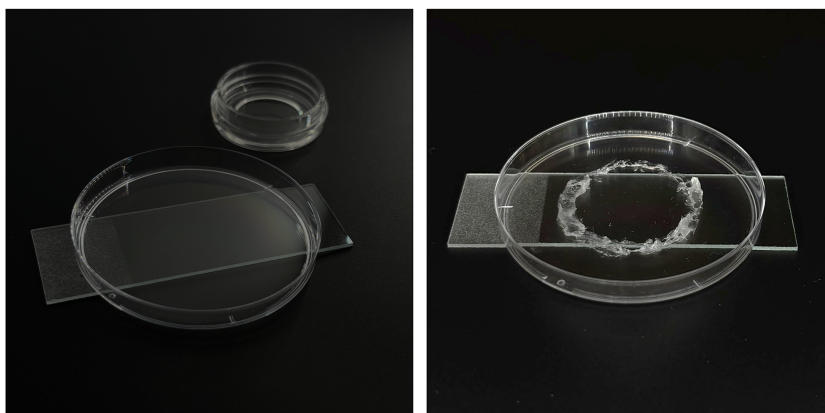


Figure 3. Mounting model and imaging dish (left) used to create a petroleum jelly reference stamp on the Petri dish lid (right)

C. Traditional mounting of embryos

Note: Here, the traditional mounting approach is detailed for the lateral orientation of zebrafish embryos.

1. Place embryos in a 4% anesthetic solution [12] for 5–10 min to ensure samples are immobilized.
2. Melt 0.8% low-gelling point agarose by heating the glass test tube in a water bath using a 50 mL beaker, microwaving in 15-s intervals.
3. Allow the agarose to cool until the glass tube can be held comfortably by hand but remains liquid.
4. Using a glass pipette attached to a pipette pump, transfer embryos into the cooled agarose and then to a 35 mm glass-bottom imaging dish.
5. Gently manipulate the embryos to a lateral orientation in the liquid agarose with a dissecting needle.

Note: Ensure that samples are flat against the glass surface to minimize the necessary working distance for imaging.

6. Allow agarose to set for 10–15 min.
7. Fill the 35 mm glass-bottom imaging dish to the very top with anesthetic solution.

D. Assemble with the mounting model

Note: The combination of the traditional mounting approach with the dual-configuration mounting model is outlined in this section.

1. Either by hand or with a wood applicator stick, line the rim of the 35 mm glass-bottom imaging dish with a thick layer of petroleum jelly (Figure 4).

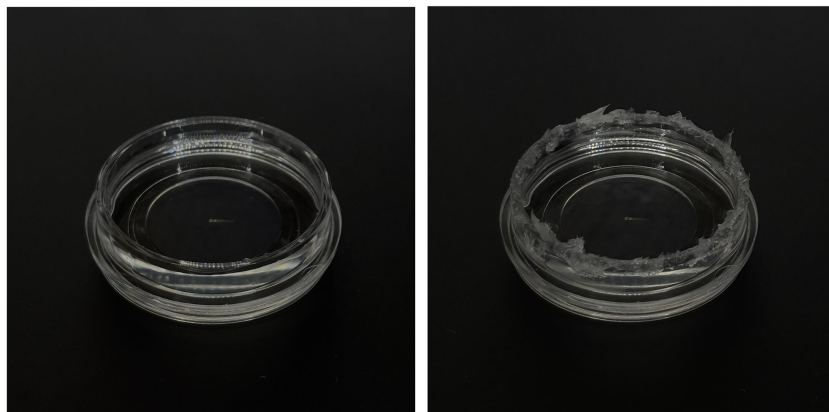


Figure 4. Glass-bottom imaging dish (left) lined with petroleum jelly (right)

2. Carefully fill the petroleum jelly-lined imaging dish to the very top with anesthetic solution.
3. Invert the dual-configuration mounting model 180° and align the petroleum ring with the petroleum jelly-lined confocal dish below. Press together to establish a hydrophobic seal (Figure 5).

Note: If there is a large air bubble, unseal the 35 mm glass-bottom confocal dish and fill with anesthetic solution until a convex meniscus forms. Then, reseal the mounting model.

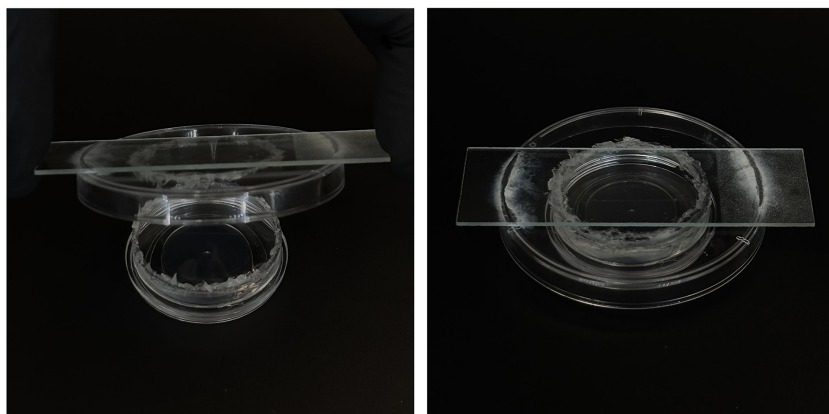


Figure 5. Placing the mounting model onto the imaging dish lined with petroleum jelly (left) to establish a hydrophobic seal (right)

4. Reinvert the mounting model (Figure 6).

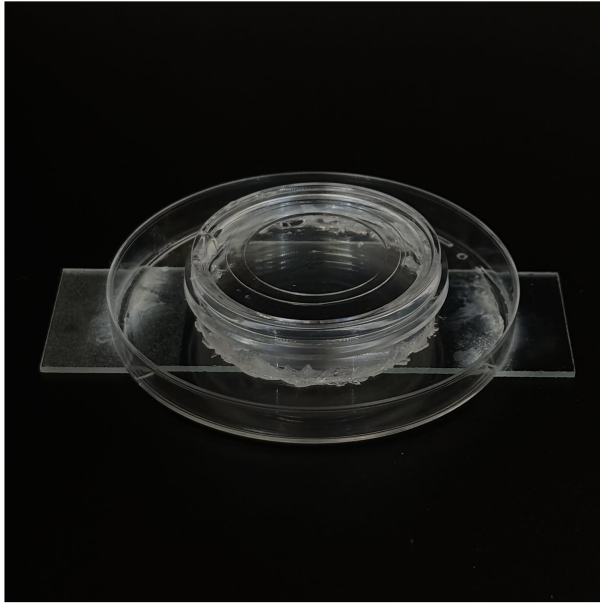


Figure 6. Mounting model ready for use on an upright microscope stage

Validation of protocol

This protocol has been routinely validated by use in our lab to conduct two-photon laser ablation experiments on an upright microscope, followed immediately by time-lapse imaging on an inverted spinning disk confocal (Figure 7). This approach enables rapid switching between imaging modalities, minimizing the time during which the effects of laser perturbation go unobserved. This capability is particularly important for visualizing debris clearance kinetics, allowing for cellular dynamics to be monitored closely from the moment of injury onward. The hydrophobic seal effectively maintains sample accessibility and stability across configurations, contributing to ease in re-identifying the same anatomical region across microscopes (Figure 8).

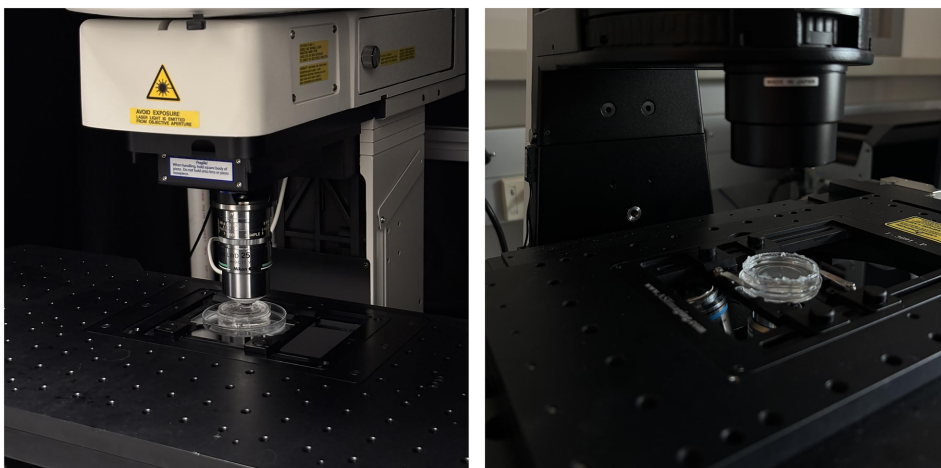


Figure 7. Mounting model demonstrated on upright two-photon (left) and inverted spinning disk confocal (right)

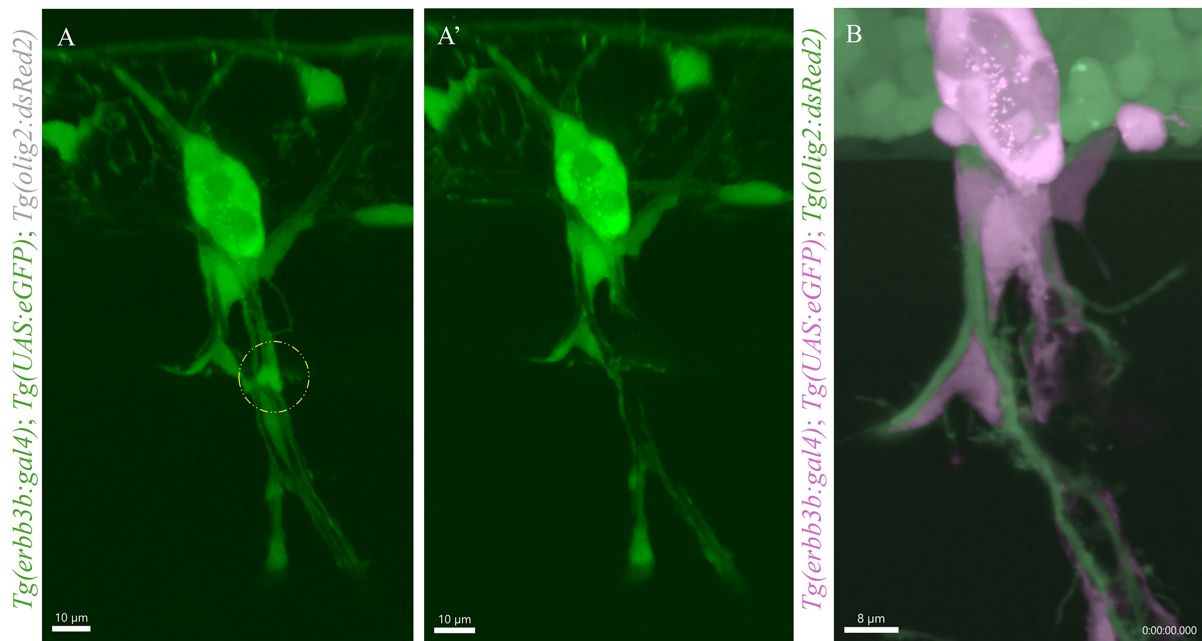


Figure 8. Protocol validation using *Tg(erb3b3b:gal4)^{nkgsaizGFPD37A}; Tg(UAS:eGFP)^{nkuaagfp1a}; Tg(olig2:dsRed2)^{vu19}* larval embryos at 3 days post-fertilization (dpf). Only the GFP channel is shown in panels A and A'. To explore the phagocytic potential of motor exit point (MEP) glia, an *erb3b3b*⁺ cell (A) along the caudal primary spinal motor neuron pathway was targeted with laser ablation (A'), as indicated by the yellow circle, using an upright two-photon microscope [13]. This anatomical region of interest was time-lapsed immediately after on an inverted spinning disk confocal (B). This approach allows for targeted focal ablation on a multiphoton microscope with limited red wavelength access, followed by faster imaging speeds and red channel accessibility on a spinning disk confocal. The confocal image in B was pseudocolored in Imaris, with dsRed2 in green and GFP in pink.

General notes and troubleshooting

General notes

While this mounting strategy offers flexibility across imaging configurations and is broadly compatible with common microscope stages, consistent repositioning of the same region of interest still requires visual landmarks like the yolk extension. This model does not change working distance requirements; however, users should still confirm appropriate clearance under upright objectives per the standard procedure.

Troubleshooting

Problem 1: A single air bubble is trapped in the 35 mm imaging dish, obscuring the sample.

Cause: Insufficient volume of anesthetic solution can cause trapped air upon hydrophobic seal.

Solution: Break the hydrophobic seal and fill the dish to maximum capacity, aiming for a convex meniscus with anesthetic solution prior to resealing.

Problem 2: Adhesive degradation between the Petri dish lid and glass slide.

Cause: Standard cyanoacrylate (super glue) forms a semi-permanent bond with glass, so re-adherence is often necessary over time.

Solutions: Re-adhere the Petri dish lid to the glass slide with super glue. Alternatively, more durable materials like UV-curable resin or modeling clay can be used. With the latter, take care to ensure the slide is as flat and flush against the dish as possible.

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The following figures were created using BioRender: Graphical overview, Murphy, H. (2026). <https://BioRender.com/x579fyx>.

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

Husbandry and generation of embryos: All animal studies were approved by the Florida Atlantic University (FAU) Animal Care and Use Committee. Embryos were raised at 28.7 °C in egg water. Zebrafish cannot be sexed until adulthood; therefore, embryos of undetermined sex were used. Pigmentation was inhibited in embryos using phenyl-thiourea (PTU) (0.003%) in egg water. All studies were conducted using stable, germline transgenic lines. Fish were housed and maintained in our fish facility at FAU under standard laboratory conditions. Adult zebrafish strains were kept at 28 °C on a 14/10 h light/dark cycle. To generate embryos, adult male and female zebrafish were housed in breeding tanks overnight and allowed to spawn naturally.

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