Chapter 9

Adeno-Associated Virus (AAV) for Tracking Neural Development and Connectivity in *Xenopus*

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Abstract

Adeno-associated viruses (AAVs) provide a versatile tool for labeling neurons in the *Xenopus* central nervous system across developmental stages. AAV-mediated transgene expression is long-lasting and stable, enabling robust labeling of neuronal populations without genomic integration and without the need to generate transgenic lines. The choice of AAV capsid and promoter can restrict expression to a population of interest, and depending on the route and timing of delivery, AAVs can selectively target either progenitor-derived neuronal cohorts or mature neuronal populations. Here, we have outlined two labeling strategies. First, intraventricular injections in tadpoles transduce neural progenitor cells lining the ventricular system, resulting in cohort-based labeling of neurons as they differentiate during development. Second, direct intraparenchymal injections in post-metamorphic frogs enable spatially restricted labeling and anterograde or retrograde tracing of connectivity within defined brain regions. Together, these approaches provide selective access to developing and mature neural circuits in *X. laevis*, supporting applications ranging from anatomical tracing to functional imaging and manipulation of neural circuits.

Keywords AAV, Neural tracing, Developmental cohorts, Connectivity, Viral injections

1 Introduction

AAVs are single-stranded DNA viruses that have transformed neuroscience research by enabling targeted, stable transgene expression in specific cell populations. While initially optimized for rodents, AAVs have recently proven effective in amphibians, including both developing and mature *Xenopus laevis* [1, 2], offering new possibilities for tracking and manipulating neuronal populations as they undergo major reorganization during metamorphosis [3, 4].

A key advantage of AAVs over traditional dye-based tracers is their ability to drive gene expression using cell-type or tissue-specific promoters [5–7], as well as intersectional genetic strategies such as the Cre/loxP system [8]. The spread and specificity of viral expression are further influenced by the AAV capsid serotype [9, 10]. In *X. laevis*, AAV5 and AAVrg (a variant optimized for retrograde

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transport [11]) efficiently transduce neurons at both tadpole and post-metamorphic stages [1], enabling new experimental approaches for neural circuit mapping and manipulation.

Recombinant AAVs are replication-deficient, exhibit low pathogenicity [12], and are generally classified as low biosafety-level vectors, making them broadly accessible for use in most research labs. The AAV genome can accommodate up to ~4.8 kb of genetic cargo [13], allowing for expression of fluorescent reporters, functional actuators (e.g., opsins and chemogenetic tools), or barcoded sequences for single-cell transcriptomics [14]. Following injection, transgene expression can be detected within 1–2 weeks, typically reaching peak levels within ~3–4 weeks, and remains stable for extended periods, making AAVs suitable for long-term studies [8].

By circumventing the need to generate transgenic lines, AAVs enable rapid visualization, ablation, functional imaging, and optogenetic manipulation of defined neuronal populations. This is particularly advantageous in *Xenopus*, where generation times make the production of stable transgenic lines time-intensive. AAVs have been screened in salamanders (*Ambystoma mexicanum* [2] and *Pleurodeles waltl* [1]) and frog species (*Xenopus laevis* and *Pelophylax bedriagae* [1]), supporting broad applicability across amphibians, although further species-specific validation and capsid screening remain necessary.

In this protocol, we describe how to deliver AAV vectors into the *X. laevis* nervous system to (1) label developing neurons in tadpoles and (2) trace neural connectivity in post-metamorphic frogs. The first approach exploits the fact that ventricular injections during early development transduce adjacent neural progenitors, which later differentiate into labeled neurons—enabling tracking of cohorts of cells based on their developmental birth time. The second approach employs AAVs for anterograde or retrograde tracing of neural projections in mature animals, allowing the mapping of regional connectivity in the frog nervous system.

2 Materials

All animal procedures should follow institutional and national guidelines for animal welfare. Use high-quality AAV preparations ($\geq 10^{13}$ GC/mL) and minimize freeze-thaw cycles, which reduce viral potency. Store AAVs at −70°C to −80°C in small aliquots (4–5 µL) for long-term use. Thaw aliquots immediately before injection and keep them on ice during the procedure.

2.1 Viral Aliquots

1. **AAVconstructs**: For constructs and promoter selection (*see* **Note 1**). In this protocol, we use two CAG-driven GFP reporters obtained from Addgene (RRID:SCR_002037), **AAV5-CAG-GFP** (Addgene #37825-AAV5) and **AAVrg-CAG-GFP** (Addgene #37825-AAVrg). Both exhibit high

neuronal tropism in *Xenopus*, with the AAV5 exhibiting predominantly anterograde spread, and AAVrg spreading in a retrograde manner (from the axon to the soma) [1].

2. **Fast Green FCF** (Sigma-Aldrich F7252): A biosafe transient dye used to visualize viral injections. Prepare a 0.5% (w/v) stock solution by dissolving 0.5 g Fast Green FCF in 100 mL sterile distilled water. Filter-sterilize, aliquot, and store at 4°C for up to 6 months. Immediately before injection, mix the stock solution with virus at a 1:5 ratio, resulting in a final Fast Green concentration of 0.1% (w/v) in the injection mixture.

2.2 Injection Apparatus

1. **PicoSpritzer injection system** (PV820, WPI)—air-pressure based system used for tadpole injection (gated injection mode).
2. **Nanoject III** (Drummond)—oil-backfilled nanoliter injector for discrete, programmable injections in juvenile and adult frogs.
3. **Glass capillaries** (3-000-203-G/X, Drummond).
4. **A capillary puller** (P-97, Sutter Instrument).
5. **Electric capillary beveler** (EG-400, Narishige).
6. **GelLoader pipette tips** – for backfilling capillaries with virus prior to mounting.
7. **Mineral oil**.
8. **Parafilm**.

2.3 Anesthesia, Aftercare, and Analgesia

1. **10× MMR+ (Marc's Modified Ringer's with EDTA)**. 58.4 g NaCl, 1.49 g KCl, 2.46 g MgSO4·7 H2O, 11.92 g HEPES, and 0.37 g EDTA dissolved in 1000 mL MilliQ H2O. Adjust pH to 7.5 with NaOH, then add 2.94 g CaCl2·2 H2O. Dilution to 0.1× results in a final pH of ~7.4.
2. **MS-222 (Tricaine methanesulfonate) for anesthesia**. Ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma, #E10521), used for recovery anesthesia of tadpoles and frogs, make fresh on the day of use. **For tadpoles**: prepare a 0.01% (w/v) solution by dissolving 0.10 g MS-222 and 0.15 g $NaHCO_3$ in 1000 mL 0.1× MMR. **For juvenile and adult frogs**: prepare a 0.015% (w/v) solution by dissolving 0.15 g MS-222 and 0.225 g $NaHCO_3$ in 1000 mL 0.1× MMR.
3. **MS-222 For deep anesthesia or euthanasia**: Prepare a 0.1% (w/v) solution by dissolving 1.0 g MS-222 and 1.5 g $NaHCO_3$ in 1000 mL 0.1× MMR. Make fresh on the day of use.
4. **After-injection bath:** Fill a recovery tank with 1 L of 0.1× MMR supplemented with gentamicin sulfate (50 μg/mL; Sigma-Aldrich, G1914-5G).

5. **Flunixin meglumine**. Used for perioperative and postoperative analgesia in juvenile and adult frog. Prepare a 4.5 mg/mL working solution in sterile 0.9% NaCl.

2.4 Surgical Tools

1. Micromanipulator for stable positioning of the injection capillary.
2. Suture kit: 5-0 (juveniles) or 6-0 (adults) VICRYL RAPIDE®.
3. Fine forceps (Dumont #5).
4. Surgical clamps.
5. Syringes (1 mL and 5 mL) with appropriate needles (31G).
6. Sugi absorbent points or equivalent for drying skull and controlling bleeding.
7. Surgical scissors for skin preparation.
8. Dental drill (Foredom) equipped with 0.75 mm carbide bit (for frog injections).

2.5 Tissue Processing and Immunodetection

1. PBS (phosphate-buffered saline, 1×). 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 in distilled water; pH adjusted to 7.4.
2. 4% paraformaldehyde (PFA) in PBS, for fixation.
3. PBS + 0.2% Triton X-100 (PBST) for washing and staining.
4. 5% sucrose/PBS with 8% cold fish skin gelatin for cryoprotection.
5. Tissue-freezing medium and molds for embedding.
6. Dry ice for cooling 2-methylbutane.
7. 2-methylbutane/isopentane (cooled on dry ice) for rapid freezing.
8. Primary antibodies: chicken polyclonal anti-GFP (RRID: AB_300798) and rabbit polyclonal anti-RFP RRID: AB_2209751) (*see* **Note 2**).
9. Secondary antibodies: donkey anti-chicken IgY, AF488 (RRID: AB_2340375) or appropriate anti-rabbit Secondary.
10. PVA DABCO antifade medium—for mounting.

3 Methods

3.1 Capillary Preparation

1. Pull several capillaries using a programmable puller and trim the tip to ~10–12 mm using fine forceps.
2. Bevel the trimmed tip at 30° on an electric beveler while continuously adding Milli-Q water to the abrasive surface to

prevent glass overheating. To supply water, use a water-filled syringe connected to the capillary using a short piece of rubber tubing (*see* **Note 3**).

3. Remove residual water from the capillary by expelling air through the capillary using either a PicoSpritzer or an air-filled syringe via flexible tubing.

3.2 Tadpole Injections

This intraventricular injection protocol has been validated in *X. laevis* tadpoles at Nieuwkoop–Faber stages 40–60 [15].

1. **Immerse** tadpoles in 0.01% (w/v) MS-222 in 0.1× MMR for several minutes until movement ceases. Transfer a tadpole to a Petri dish lined with gauze soaked in 0.01% (w/v) MS-222 and keep it moist throughout the procedure.
2. Prepare the **injection** mixture by mixing AAV with 5× Fast Green stock at a 1:5 ratio (e.g., 1 µL Fast Green with 4 µL viral preparation). Keep the mixture on ice.
3. Load the virus/Fast Green mixture into the capillary using a GelLoader pipette tip immediately before injection.
4. Mount the capillary onto the PicoSpritzer and eject a test droplet.
5. Calibrate the drop size (~10 nL per pulse) using the eyepiece scale of the stereomicroscope (4–5 units on the scale at 2× magnification).
6. Gently hold the anesthetized tadpole. Orient the animals so that the intended injection region is accessible.
7. Insert the capillary into the appropriate region depending on the labeling strategy:
   – **Brain ventricle**: penetrate the skin along the dorsal midline and enter the ventricular space at the level of the third ventricle (Fig. 1).
   – **Spinal canal**: insert the capillary through dorsal trunk muscles and lower it into the central canal.
8. Set the PicoSpritzer to gated mode and deliver four pulses (~40–60 nL total) of virus mixture. The ventricular system typically becomes visibly green upon successful injection.
9. After injection, return tadpoles to fresh 0.1× MMR and monitor until recovery (*see* **Note 4**).

3.3 Frog Surgery, Virus Injection, and Postoperative Care

3.3.1 Analgesia and Anesthesia

1. Weigh the animal to calculate the appropriate dose of flunixin (25 mg per kg body weight).

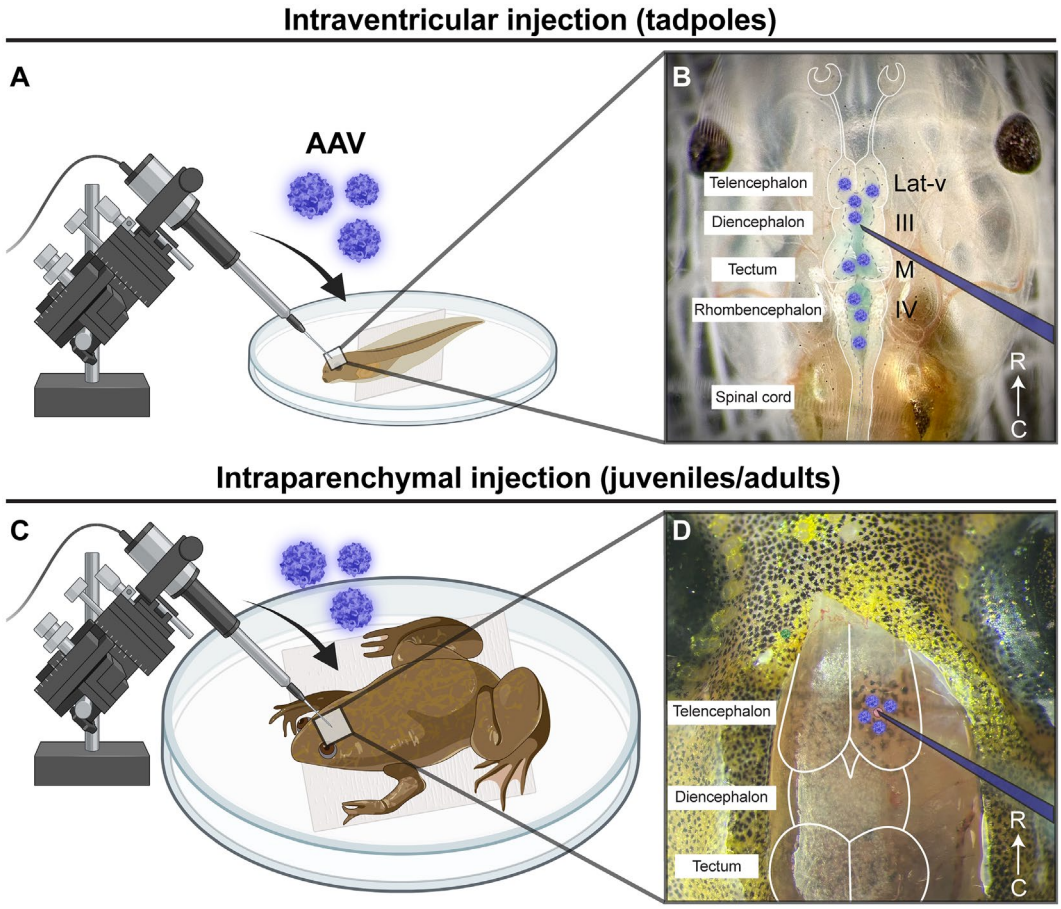

Fig. 1 AAVdelivery strategies for labeling neural development and connectivity in *Xenopus*. (**A, B**) Intraventricular AAV injections in tadpoles label neural progenitors lining the ventricular system, resulting in cohort-based labeling of neurons as they differentiate. (**C, D**) Intraparenchymal AAV injections in juvenile and adult frogs target defined brain regions for local circuit tracing. Schematics illustrate injection approaches, and representative images show corresponding anatomical targeting. Ventricular abbreviations: *Lat-v* lateral ventricle, *III* third ventricle, *IV* fourth ventricle, *M* midbrain ventricle. R–C indicates the rostrocaudal axis. Created in BioRender. Sweeney, L. (2026) https://BioRender.com/31a9dho

2. Place the frog on a clean, dry surface, and gently wrap it in moist gauze to immobilize the body, leaving the dorsal surface exposed (cut a small opening in the gauze if necessary).
3. Inject flunixin subcutaneously along the dorsal body wall at an angle of ~45°, gently lifting the skin with the needle to avoid muscle injury.
4. Pause 10 s before withdrawing the needle to minimize leakage.
5. Return the frog to an isolated tank and wait for 30 min for the analgesic to take effect.
6. Anesthetize the frog by immersion in 0.015% (w/v) MS-222 until deep anesthesia is achieved (complete loss of withdrawal reflexes).

7. Transfer the anesthetized frog to the surgical scope, wrap the body in MS-222–soaked gauze, and position it with the head exposed and slightly elevated.

3.3.2 Expose the Target Region

1. Pick up the skin above the region of interest (Fig. 1) using forceps and make an incision with fine scissors.
2. Dry the exposed skull with absorbent points and avoid damaging blood vessels.
3. Manage any bleeding by gently pressing with absorbent points.
4. By moving a dental drill into a circular motion, drill a hole in the skull directly above the brain area of interest. Drill in short intervals, keeping the bone moist, and stop once the skull feels soft under gentle pressure.
5. Very carefully remove the bone fragment and meninges using fine forceps to expose the brain surface.

3.3.3 Injection

1. Backfill a beveled capillary with mineral oil and mount it onto the Nanoject.
2. Place ~2 μL of AAV mixture on a piece of parafilm. Fill the capillary by lowering it into the droplet and activating the "Fill" function in the "Manual" menu.
3. Program the Nanoject to deliver 1 nL per pulse at a 1 nL/s rate with 100 cycles and a 12 s delay. Test droplet formation before proceeding.
4. Position the capillary over the craniotomy and slowly lower it into the target brain region (Fig. 1). Start the injection program and, during delivery, switch off the microscope's illumination to prevent drying of the brain and eyes. Typical injection volumes of 100 nL are delivered over ~20 min.
5. Upon completion, leave the capillary in place for 5 min to minimize backflow.
6. Withdraw the capillary slowly in stages.

3.3.4 Wound Closure and Recovery

1. Suture the skin using a 5-0 (juveniles) or 6-0 (adults) kit. Tie double knots and trim excess thread. Ensure the incision is closed without trapping air.
2. Prepare 1 L of 0.1× MMR containing gentamicin and fill a recovery tank. Wrap the animals in MMR–soaked gauze and place it on a weighing boat. Float the boat in the tank so the frog's head remains above water until it regains consciousness and enters the water on its own.
3. Monitor the animal until it regains spontaneous movement (~1.5 h). Repeat the flunixin analgesia 24 h after surgery. House the frog individually in 0.1× MMR, change water two to three

times per week, and feed normally. Allow 21–28 days for maximal AAV expression before tissue collection (*see* **Note 5**).

3.4 Tissue Processing and Immunodetection

1. **Fixation**: Sacrifice animals 21 days post injection. For tadpoles, euthanize in 0.1% MS-222, dissect out the brain and fix by immersion in 4% PFA on ice for 1.5 h. For juveniles and adults, deeply anesthetize in 0.1% MS-222 until complete cessation of reflexes. Decapitate the frog and remove the skull. Fix the isolated brain in 4 % PFA overnight at 4°C.
2. **Cryoprotection:** Wash fixed tissue three times in 1× PBS. Cryoprotect overnight at 4°C in 15% sucrose/PBS with 8 % cold fish skin gelatin.
3. **Embedding**: Lightly dry juvenile tissue and embed it in tissue freezing medium in cryomolds. Freeze in 2-methylbutane cooled on dry ice.
4. **Sectioning**: Section at 40 μm using a cryostat. Let them dry at room temperature for an hour and store at −70°C to −80°C.
   a. **Immunodetection**:
5. Take out the slides from the freezer and dry at 4°C overnight.
6. Wash twice in 1× PBST.
7. Add 900 μL of primary antibody per slide (e.g., chicken anti-GFP, 1:2000) diluted in PBST. Include anti-RFP if detecting red reporters.
8. Incubate overnight at 4°C.
9. Wash sections three times in 1× PBST.
10. Add 900 μL secondary antibody per slide diluted in PBST.
11. Incubate at room temperature for 40 min.
12. Wash three times in PBST.
13. Mount coverslips using 85 μL of PVA DABCO antifade medium.
14. Leave for at least an hour to air dry before storing away or imaging.
15. Imaging: Acquire z stacks (30–40 optical planes with 1.5 μm steps) using a confocal microscope (see Fig. 2).

4 Notes

1. Choice of promoter. The ubiquitous CAG promoter provides robust expression across neuronal populations. AAV5 yields strong anterograde labeling, whereas AAVrg enables retrograde

transduction from axon terminals to somata (see Fig. 2). Cell type- or tissue-specific enhancers can be used to further restrict viral expression.

2. The AAV-driven signal can be observed in vivo, making it suitable for functional imaging. For analysis in fixed tissue, signal amplification using antibodies is recommended to enhance detection sensitivity and image quality.
3. Producing a sharp bevel at the capillary tip is essential for successful viral injection. A well-beveled tip facilitates entry into the tissue, minimizes tissue damage and reduces likelihood of excessive immune responses. If an electric beveler is not available, the capillary tip can be carefully sharpened using fine-grit sandpaper.
4. Intraventricular injections in tadpoles are minimally invasive and typically have near-complete survival. When using AAV5, labeling efficiency is typically also close to 100%.
5. Juvenile and adult frogs generally recover well from surgery. Skin incisions typically close within 1 week, after which absorbable sutures dissolve without intervention. Labeling efficiency depends on user experience; therefore, plan for at least twice the number of animals required to obtain the desired number of successfully labeled specimens. With experience, injection success rates can increase to ~80%.

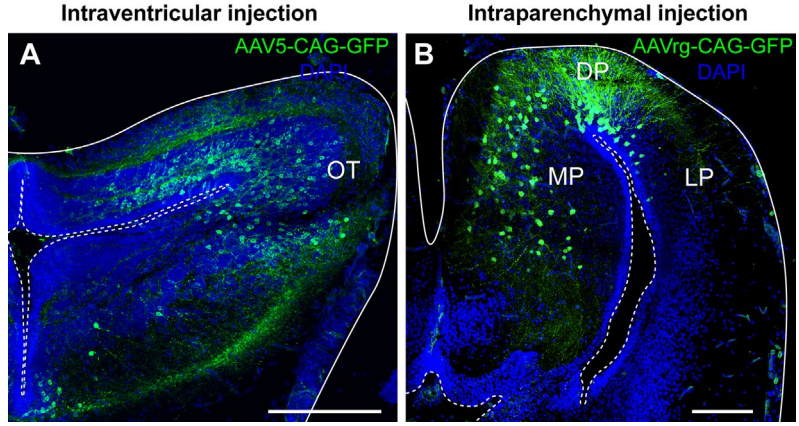

Fig. 2 Representative outcomes of AAV-mediated neuronal labeling in *X. laevis*. (**A**) Intraventricular injection of AAV5-CAG-GFP in tadpoles results in widespread labeling of neurons derived from ventricular progenitors, illustrated here in the optic tectum (OT). (**B**) Intraparenchymal injection of AAVrg-CAG-GFP in juvenile frogs produces localized retrograde labeling of projection neurons surrounding the injection site in the pallium. GFP signal is shown in green and DAPI nuclear counterstain in blue. Dashed lines indicate ventricular spaces. *DP* dorsal pallium, *MP* medial pallium, *LP* lateral pallium. Scale bars 200 μm

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