

Aug 08, 2025

Version 8

RNAscope Multiplex Fluorescent V2 Labeling of frog brains V.8

 Version 1 is forked from [Preparation and RNAscope-labeling of fresh mouse midbrain tissue](#)

DOI

<https://dx.doi.org/10.17504/protocols.io.261ge874dg47/v8>

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Protocol Citation: Billie Goolsby, A. Zach Reddy, Melody Joy Dailey, Lauren O'Connell, Adithi Rao 2025. RNAscope Multiplex Fluorescent V2 Labeling of frog brains. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.261ge874dg47/v8> Version created by **Billie Goolsby**

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Protocol status: Working

We use this protocol and it's working

Created: August 08, 2025

Last Modified: August 08, 2025

Protocol Integer ID: 224288

Keywords: rnascope, fresh tissue, sectioning, rnascope multiplex fluorescent v2 labeling of frog brain, rnascope multiplex fluorescent v2 labeling, coronal sections of frog brain, frog brain, labeling coronal section

Funders Acknowledgements:

Gilliam Fellowship

Grant ID: GT15685

NIH R01

Grant ID: DP2HD102042

Disclaimer

While this guide has generally worked for the authors, one protocol does not fit all experiments. This is a launching place. To have the best results possible, you should actively research your genes of interest, develop your methods to answer your research question, engage with and modify the protocol as needed, and consult with ACD before beginning.

Abstract

Below we describe preparing and labeling coronal sections of frog brains.

Attachments



[UM 323100 Multiplex ...](#)

2.8MB

Materials

Part A:

	A	B
	Material	Supplier and Catalog Number
	RNaseZap	Invitrogen (AM9780)
	Scissors	Fine Science Tools (14084-08)
	Forceps	Fine Science Tools (11242-40)

Part B:

	A	B
	Material	Supplier and Catalog Number
	Cryostat	Leica (CM1860)
	Anti-roll plate	Leica (14047742497)
	Brushes	Electron Microscopy Sciences (66100-30)
	OCT Medium	Sakura (4583)
	Superfrost Plus Microscope Slides	Thermo Fisher Scientific (12-550-15)

Part C & D:



A	B
Material	Supplier and Catalog Number
Probes	RNAscope Target Probes (Catalog or Made-to-Order C1 to C3 Probes, not compatible with the RNAscope probes with T1-T3 designation).
RNAscope Probe Diluent	Cat. No. 300041
RNAscope Multiplex Fluorescent Reagent Kit v2 (includes Pretreatment Kit, detection kit, wash buffer and TSA dilution buffer)	Cat. No. 323100
Wash Buffer	Advanced Cell Diagnostics (310091)
HybEZ Oven	Advanced Cell Diagnostics (310010)
Pretreatment Kit (Protease & hydrogen peroxide)	322340 RNAscope™ Protease III & Protease IV Reagents 322330 RNAscope™ H2O2 & Protease Plus Reagents
UltraPure DNase/RNase-Free Distilled Water	Invitrogen (10977049)
Wash containers	Mortech Manufacturing (SP233)
Safe Lock Centrifuge Tube, 1.5 mL	Eppendorf (0030123611)
ImmEdge Hydrophobic Barrier PAP Pen	Vector Laboratories (H-4000)
Prolong Gold Antimountant	Thermo Fisher (P36930)
Coverslip	Corning (2980-225)
Falcon 50 mL Conical Centrifuge Tubes	Fisher Scientific (352098)

Before you begin

- 1 **Please keep in mind that this protocol is designed for fresh frozen, adult frog brains.** Tadpoles are much smaller and can be dissected within their skull cap, or have their brains dissected out. Think about how much the PFA will actually penetrate your brain tissue - is your brain only lightly fixed, very fixed, or fresh? Make sure you are thinking thoroughly about your experimental design before beginning the protocol. **This protocol is not failproof, and no experiment is exactly the same. For this reason, no individual author should be held responsible if it does not work as expected. Pilot and quality-check your experiment before using real samples.**

This protocol uses the fresh frozen method for tadpoles dissected with the skull cap intact and placed in PFA, as well as for adult brains that are snap frozen. However, other pretreatments may also be equally effective. These alternatives are detailed in the ACD manual, and free consultations with technical representatives are available.

Other types of dissections (such as decalcification) can impact the integrity of DNA and RNA, which are necessary for in situ hybridization (ISH) techniques. While acid-based decalcification can be rapid, it may also damage nucleic acids, making them unsuitable for ISH. EDTA-based decalcification is generally preferred as it preserves DNA and RNA better, allowing for more reliable ISH results.

A. Frog brain extraction

- 2 Before you begin, spray experimenter's gloves and all required tools with RNaseZap before you begin, as well as between brain extractions. Wipe the RNaseZap away with RNase free water or ethanol, as RNaseZap contains SDS, which can degrade samples.

Alternative: for PFA fixation, prepare 4% PFA and place on ice.

- 3 Prepare an eppendorf tube for embedding the brain by labeling it, cutting off the conical tip, closing the lid, and filling 1/3 of the tube with OCT. Place on dry ice to begin solidification.

Alternative: put ~500 uL of 4% PFA in an eppendorf tube, and put on regular ice.

- 4 After following APLAC procedures for anesthesia, decapitate the frog and extract the brain from the skull. Gently cut the skull casing along the side so that the skull capsule containing the brain is exposed, but the brain itself is uncut. Using two sets of forceps, pull part the skull casing, exposing the brain. Gently remove the brain with forceps (careful - optic nerves may still be attached at the bottom of the brain and may need to be severed with microscissors or fine forceps).

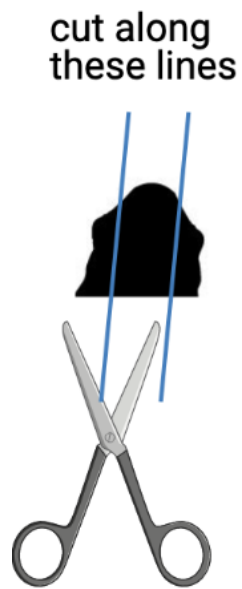


Image courtesy of Biorender.

- 5 After the OCT has semi-hardened, remove from dry ice and fill the tube to 2/3 with fresh OCT. Embed the brain with the caudal side facing the lid of the tube (which ensures you begin sectioning rostrally). Make sure the brain is aligned in the tube and leave to harden.

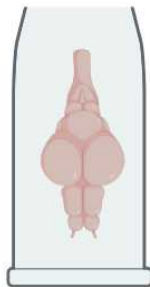


Image courtesy of Biorender.

Alternative: Put brain/tissue in PFA eppendorf tube. Let fix overnight. After 24 hrs, move brain to a new tube, replace 4% PFA with 1X PBS (RNase-free).

After 24 hrs, move the brain to a new tube, fill it with 30% sucrose and place in the 4°C fridge to avoid bacterial/fungal growth on your sample. After 24 hrs following this, remove the brain and embed in OCT as described above. **If you do a PFA fixation on samples other than tadpoles that still have their heads (as described here), you are performing a new protocol that requires your own development.** In that case, please refer to the ACD manual.

- 6 **Alternative:** Immediately snap freeze the brain by placing the tube on dry ice. Make sure the brain aligns correctly as it freezes.
- 7 Frozen brains can be moved to either an RNase-free cryostat at -18°C or stored at -80°C.

B. Sectioning

- 8 Set the temperature of the cryostat objective and chamber to -18°C.
- 9 Clean the interior of the cryostat with 100% ethanol.
- 10 Before sectioning, spray the experimenter's gloves and tools with RNaseZap. Tools that come into direct contact with the sections (e.g. brushes, anti-roll plate) should be air dried completely before being placed in cryostat. Do not rub your hands as you will cause static electricity that can affect your sections.
- 11 Place the culture tube with the brain in the cryostat chamber and let it equilibrate to chamber temperature for about 10 minutes.
- 12 For coronal sections, mount the caudal end of the brain to the chuck using OCT mounting medium. Mount the brain as perpendicular to the horizontal plane of the chuck as possible. Equilibrate to chamber temperature again for about 10 minutes.
- 13 Place the chuck with the brain on the objective and adjust its orientation to make the brain perpendicular to the blade. As you begin cutting, adjust the position of the sample so the brain landmarks (optic tectum) are as symmetrical as possible. Ensure that the anti-roll plate is positioned correctly before reaching your area of interest.
- 14 Cut coronal brain sections serially at 15-18 µm on a cryostat throughout the rostro-caudal extent of the region of interest (ROI).



- 15 Directly mount sections onto Superfrost glass slides. Mount the sections within the white cross hatches of the slide only.
- 16 Air-dry your slides at room temperature (RT) for 10-20 minutes, and then store at -80°C in a slide box within an airtight container (e.g. Ziplock bag), to avoid damaging your samples from condensation.

C. RNAscope assay Day 1

- 17 Turn on HybEZ™ Oven (ACD) and set to 40°C.
- 18 Warm the probes in a heat-bath for 10 minutes at 40°C, then cool to RT.
- 19 Make the probe mixture in an RNase free Eppendorf tube by pipetting (with filter pipette tips) 50 parts C1 probe or diluent, 1 part C2 probe (optional) and 1 part C3 probe (optional). Prepare about 150 µL of probe mix per slide, assuming each slide contains ~20-25 brain sections.
- 20 Fill a styrofoam box with dry ice. Remove the slide box from the -80°C, remove from airtight container, and place on dry ice while taking out slides to be stained. Immediately return the box to the airtight container and place back in the -80°C.

Safety information

Dry ice, while useful for cooling, requires careful handling due to its extreme cold and the potential hazards of the carbon dioxide gas it releases. Always wear insulated gloves and eye protection when handling dry ice.

- 21 Lay the slides flat, tissue side up, on a clean surface. Fix the slides in RNase-free 4% PFA/1x PBS at RT for 60 minutes. Keep the slides covered to minimize fumes.

Take out the ACD hydrogen peroxide and protease kit to get it up to room temperature.
- 22 Following the 60 minute period, wash the slides twice in RNase-free 1x PBS (~30 seconds per wash).
- 23 Dehydrate the slides flat in 50% EtOH in DI water for 5 minutes.



- 24 Dehydrate slides flat in 70% EtOH in DI water for 5 minutes.
- 25 Dehydrate slides flat in 100% EtOH in DI water for 5 minutes and **repeat this step 2x**.
- 26 Air dry the slides flat for 5 minutes.
- 27 Use the hydrophobic pen to draw a barrier around your sections, which will reduce reagent loss. Let air dry for 5 minutes.
- 28 Put slides into the slide holder. Have the white label of the slide facing outward from the center of the slide rack so that you can easily access the tissue on your slide.

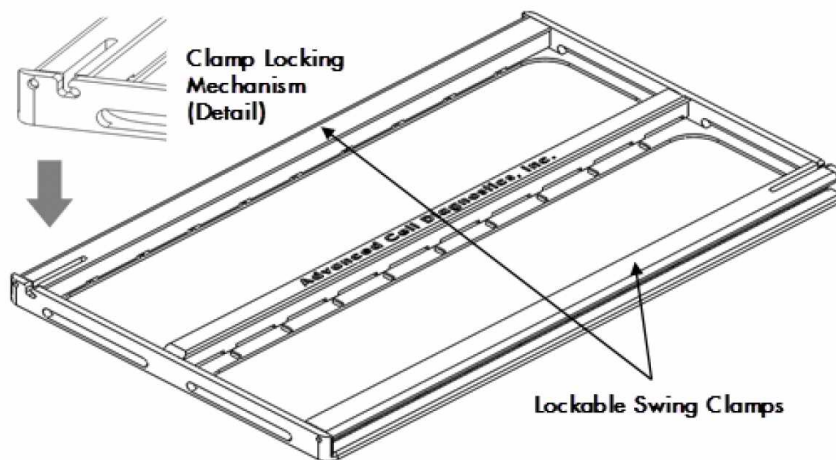


Image courtesy of ACDBio.

- 29 Incubate the slides flat with hydrogen peroxide at RT for 15 minutes. Keep the slides covered to prevent dust contamination.

Note: If you run out of hydrogen peroxide, you can make 3% H_2O_2 in the lab. Take 1 mL of 30% H_2O_2 and fill to 10 mL with DI H_2O .

- 30 Rinse the slides in RNase-free DI water (not milliQ) at RT, twice. Do this step with the slides flat.





31 Incubate the slides flat with Protease IV at RT for 30 min. Keep the slides covered to prevent dust contamination.



32 Rinse slides in RNase-free DI (not milliq) water at RT, twice. Do this step with the slides flat.



33 Decant excess liquid from the slides by gently tapping the slide edge on a paper towel. Pipette enough probe mixture to cover sections (~150 uL) while also ensuring that you have enough remaining for all your slides.

34 Incubate for 2 hours in the HybEZ™ Oven at 40°C.



35 Wash in 1X wash buffer for 2 minutes, twice.



36 **This step is optional and can be skipped. If you do skip it, you cannot stop the protocol until it is complete.**



Prepare 200 mL of 5X SSC by diluting 50 mL of 20X SSC with 150 mL distilled water. 20X SSC is available online.

Fill wash tray with 5X SSC, and place slide holder inside. Make sure all the slides are covered and that the wash tray is covered with a lid. Leave overnight at RT.

D. RNAscope assay Day 2

37 **Prepare fluorophore dilutions.**

We use 520, 570, and 650 TSA fluorophores. These are approximately equivalent to 488, 555, and 647 in IHC, respectively. These fluorophores are specific for RNAscope, and are not meant to be used for any other experiment. There are ~10 uL aliquots of these fluorophores in the -80°C. Take one aliquot out and keep it in your 4°C until you have finished using it. It will remain solid at 4°C, as the fluorophores are reconstituted in DMSO.

Do not mix the fluorophores. Assign one fluorophore to one gene channel (C1,C2,C3). Keep track and write it down.

We recommend starting with a dilution of 1:1500 for TSA Vivid 520 and 570, and 1:5000 for 650, and adjusting the dilution based on signal intensity. Optimal



fluorophore dilutions may vary based on sample, target expression levels, and imaging system. Dilute in TSA buffer.

Example: For C1, I will use the 520 fluorophore. I take out the fluorophore from my 4°C, and let it reach RT so that the DMSO turns to liquid. I will add 1.26 uL of the 520 fluorophore to 1,800 uL of TSA buffer. I will use it at step 47. And so on and so forth...

38 If you stored your slides in SSC, wash in 1X wash buffer for 2 minutes, once. If you skipped the SSC step, skip this step also.



39 Decant excess liquid from the slides and add enough AMP1 to fully cover the sections. Incubate for 30 minutes in the HybEZ™ Oven at 40°C.

40 Wash in 1X wash buffer for 2 minutes, twice.

41 Decant excess liquid from the slides and add enough AMP2 to fully cover the sections. Incubate for 30 minutes in the HybEZ™ Oven at 40°C.

42 Wash in 1X wash buffer for 2 minutes, twice.

43 Decant excess liquid from the slides and add enough AMP3 to fully cover the sections. Incubate for 15 minutes in the HybEZ™ Oven at 40°C.

44 Wash in 1X wash buffer for 2 minutes, twice.

45 Decant excess liquid from the slides and add enough HRP-C1 to fully cover the sections. Incubate for 15 minutes in the HybEZ™ Oven at 40°C.

46 Wash in 1X wash buffer for 2 minutes, twice.

47 Add diluted fluorophore for labeling the C1 probe and incubate for 30 minutes in the HybEZ™ Oven at 40°C.

48 Wash in 1x wash buffer for 2 minutes, twice.



- 49 Add HRP blocker to the slides and incubate for 15 minutes in the HybEZ™ Oven at 40°C.
- 50 Wash in 1x wash buffer for 2 minutes, twice.
- 51 Decant excess liquid from the slides and add enough HRP-C2 to fully cover sections. Incubate for 15 minutes in the HybEZ™ Oven at 40°C.
- 52 Wash in 1X wash buffer for 2 minutes, twice.
- 53 Add diluted fluorophore for labeling the C2 probe and incubate for 30 minutes in the HybEZ™ Oven at 40°C.
- 54 Wash in 1x wash buffer for 2 minutes, twice.
- 55 Add HRP blocker to the slides and incubate for 15 minutes in the HybEZ™ Oven at 40°C.
- 56 Wash in 1x wash buffer for 2 minutes, twice.
- 57 Decant excess liquid from the slides and add enough HRP-C3 to fully cover the sections. Incubate for 15 minutes in the HybEZ™ Oven at 40°C.
- 58 Wash in 1x wash buffer for 2 minutes, twice.
- 59 Add diluted fluorophore for labeling the C3 probe and incubate for 30 minutes in the HybEZ™ Oven at 40°C.
- 60 Add HRP blocker to the slides and incubate for 15 minutes in the HybEZ™ Oven at 40°C.
- 61 Wash in 1x wash buffer for 2 minutes, twice.



E. Counterstain and mounting

- 62 **Before you start:** Do this procedure with no more than five slides at a time.
- 63 Decant excess liquid from the slides and add enough DAPI to fully cover each section and incubate for 30 seconds at RT.
- 64 Remove DAPI by tapping or flicking the slides. Immediately place 1-2 drops of ProLong Gold Antifade mountant on each slide and ensure that there are no bubbles.
- 65 Carefully place the coverslip on the tissue section, once again avoiding air bubbles.
- 66 Dry the slides overnight in the dark at RT.
- 67 Seal the edges of your slides with clear nail polish to prevent the coverslip moving when imaging.
- 68 Store the slides in the dark at 2-8 °C.

Protocol references

https://acdbio.com/sites/default/files/UM%20323100%20Multiplex%20Fluorescent%20v2%20User%20Manual_0.pdf

<https://www.youtube.com/watch?v=vAz3MhtrFtk>

<https://www.youtube.com/watch?v=-6uRiqzI3GM>

Acknowledgements

We gratefully acknowledge William Conrad at UC San Diego who wrote out on Protocols.IO a similar protocol for mice, and from which this protocol was forked and specified for frogs.