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Whole-mount RNA *in situ* hybridization and immunofluorescence of *Xenopus* embryos and tadpoles

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Abstract

A major advantage of experimentation in *Xenopus* is the ability to query the localization of endogenous proteins and RNAs *in situ* in the entire animal during all of development. Here I describe three variations of stainings to visualize mRNAs and proteins in developing *Xenopus* embryos and tadpoles. The first section outlines a traditional colorimetric staining for mRNAs that is suitable for all stages of development, and the second extends this protocol for fluorescence-based detection for higher spatial and quantitative resolution. The final section details detection of proteins by immunofluorescence, optimized for tadpole stages but widely applicable to others. Finally, optimization strategies are provided.

Materials

Before beginning, consult relevant Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling, use, and disposal of hazardous materials used in this protocol.

Recipes: See the end of this protocol for recipes indicated by <R>.

Reagents

Colorimetric RNA *in situ* hybridization

Acetic Anhydride (Sigma 320102)

Anti-digoxygenin-AP Antibody (Sigma 11093274910)

Alkaline Phosphatase Buffer <R>

BMB Blocking Solution <R>

BM Purple Reagent (Sigma 11442074001)

Bouin's Fixative <R>

Buffered Ethanol <R>

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Bleaching Solution <R>

Formaldehyde (37%) (Fisher MK501602)

Hybridization Buffer <R>

Maleic Acid Buffer (MAB) <R>

MEMFA fixative <R>

Methanol (Fisher A4544)

Molten agarose (1%, optional)

Paraformaldehyde (4% in PTw) <R>

PBS with Tween 20 (PTw, 0.1%) <R>

Phosphate-buffered saline (PBS, pH 7.4) <R>

Proteinase K solution <R>

RNAse A (20 μg/ml) (Fisher EN0531)

RNAse T1 (10 μg/ml) (Fisher EN0542)

SSC Buffer (20X) <R>

Triethanolamine (0.1M, pH 7–8) (Sigma T1502)

Fluorescent RNA *in situ* hybridization by hybridization chain reaction (HCR)

Acetic Anhydride (Sigma 320102)

Amplification Buffer <R>

Dextran Sulfate (50%) <R>

Formaldehyde (37%) (Fisher MK501602)

Hybridization Buffer (30%) <R>

MEMFA fixative <R>

Methanol (Fisher A4544)

Molten agarose (1%, optional)

Paraformaldehyde (4% in PTw) <R>

PBS with Tween 20 (PTw, 0.1%) <R>

Phosphate-buffered saline (PBS, pH 7.4) <R>

Probe Wash Buffer (30%) <R>

Proteinase K solution <R>

SSCT (5X) <R>

Triethanolamine (0.1M, pH 7–8) (Sigma T1502)

Immunofluorescence

CAS-Block (Invitrogen 00–8120)

Molten agarose (1%, optional)

Paraformaldehyde (4% in PBS) <R>

Phosphate-buffered saline (PBS) <R>

PBS with Triton-X100 (PBT, 0.1%) <R>

Equipment

Aluminum foil

Baskets (Figure 1A)

Only if using basket format

Made from 1.7 ml tubes and 300 μm nylon mesh (Spectra mesh 146487)

For dissected tissue, use finer mesh

See (Sive et al. 2007a) for how to make baskets

Basket racks (Figure 1B)

Only if using basket format

3D printer files are available at willseyfroggers.org/resources

Basket racks can also be made by cutting the bottoms off of 1.5 mL tube racks

Culture plates (48 wells, Fisher 720086) (Figure 1D)

Only if using basket format

Culture tubes (15 mL, Fisher 1496215E) (Figure 1C)

Only if using basket format

Forceps

Glass dishes for washes (Figure 1B)

Only if using basket format

Small (fits 24 baskets, holds 50 mL wash buffer, Wheaton Inc 900170)

Medium (fits 30 baskets, holds 100 mL wash buffer, Wheaton Inc 900203)

Large (fits 60 baskets, holds 150 mL wash buffer, Grainger 49WF37)

Glass slides and coverslips

Imaging stamps (Figure 3A)

3D printer files available at willseyfroggers.org/resources

Lateral shaker (basket format) or Nutator (tube format) at room temperature

Light box

Bright lights from a stereoscope can be substituted

Vacuum grease

Syringes (1 mL for dispensing vacuum grease onto glass slides for imaging)

Water bath (37°C) with lateral agitation

Water bath (60°C) with lateral agitation

Method

The methods below have been optimized for higher-throughput staining (24–60 samples processed in parallel), with each sample in a basket within a large rack in a glass staining dish, sharing a common buffer solution (Figure 1) (Sive et al. 2007a). This protocol is also effective, although lower-throughput, in individual vials or tubes with manual washes. Notes are provided where modifications should be made.

Colorimetric whole-mount RNA *in situ* hybridization

This section describes whole-mount RNA *in situ* hybridization with colorimetric detection by BM Purple staining (Figure 2A). This is a cost-effective strategy for assaying mRNA expression in embryos and tadpoles of all stages using *in vitro* transcribed digoxygenin-11-UTP-labeled RNA probes (Sive et al. 2007b). Heating probes at 60°C for several hours in hybridization buffer can help with penetration. Staining in parallel with a control probe (either sense-transcribed, or a probe with a known, very specific pattern) is desired.

Note: All steps are done on a lateral shaker with light agitation (~40 rpm).

Fixation & Dehydration [2.5 hours]

1. Fix animals for 2 hours at room temperature in 1X MEMFA solution.

   Note: This step and the following can be done in basket format or in individual vials or tubes if planning on long term storage before staining.

2. Dehydrate into methanol, wash several times in methanol, and freeze at −20°C at least overnight.

   Note: Samples can be stored here long term at −20°C.

Rehydration & Permeabilization [55 minutes]

3. Rehydrate step-wise into PTw: 5 minutes in 100% methanol, 5 minutes in 75% methanol / 25% H_2O, 5 minutes in 50% methanol / 50% H_2O, 5 minutes in 25% methanol / 75% PTw.

4. Wash 4 × 5 minutes in PTw.

5. Permeabilize in proteinase K solution for 5 minutes at room temperature.

   Note: For staining of superficial structures like epidermal cilia, omit this step. For staining of deeper structures, this step can be extended with careful testing

or combined with dissection for further permeabilization. Otherwise, this step should be carefully monitored and not prolonged.

Blocking & Hybridization [2.5 hours for X. tropicalis, 7.5 hours for X. laevis]

6. Wash 2 × 5 minutes in 0.1M triethanolamine (pH 7–8).
7. Wash 2 × 5 minutes 0.1M triethanolamine with acetic anhydride (125 μl acetic anhydride per 50 mL 0.1M triethanolamine).
8. Wash 2 × 5 minutes in PTw.
9. Refix for 20 minutes in 4% paraformaldehyde in PTw.
10. Wash 5 × 5 minutes in PTw.

    Note: For fluorescence-based detection, transfer here to the next section.
11. Prehybridize in hybridization solution for 1 hour (*X. tropicalis*) or 6 hours (*X. laevis*) at 60°C with shaking.

    Note: Depending on the probe, this can be shortened to 1 hour for X. laevis samples. Dissected tissues may also require less time than whole embryos.
12. Transfer into 1 μg/ml probe solution diluted in hybridization buffer overnight at 60°C with shaking.

    Note: Save prehybridization solution to reuse the next day in Step 14. For basket format, baskets are removed from the rack and placed into 15 mL round bottom culture tubes with 500 μL of probe solution each (Figure 1C). This allows each sample to have a different probe, if desired.

Probe Detection [Time to antibody incubation is 4.5 hours; antibody incubation can be done overnight at 4°C or 4 hours at room temperature; MAB washes can be done overnight at 4°C or 5 hours at room temperature; AP buffer washes take 15 minutes; developing the stain in BM Purple varies from 1 hour to days depending on the probe and sample]

Note: This section is essentially an antibody staining against Digoxygenin-11-UTP present in the RNA probe followed by enzymatic colorimetric detection. This can be modified depending on the probe label and desired detection modality.

13. Remove probe and save at −20°C for reuse.
14. Wash in hybridization buffer, reused from step 11, at 60°C for 5 minutes.
15. Wash 2 × 3 minutes in 2X SSC at 60°C.
16. Wash 3 × 20 minutes in 2X SSC at 60°C.
17. Incubate 30 minutes at 37°C in 2X SSC with 20 μg/mL RNase A and 10 μg/mL RNase T_1.
18. Wash once 10 minutes at room temperature in 2X SSC.
19. Wash 2 × 30 minutes in 0.2X SSC at 60°C.

20. Wash 2 × 10 minutes in MAB at room temperature.

21. Incubate in 2% BMB blocking solution for at least 1 hour at room temperature.

22. Incubate in antibody solution (Anti-digoxygenin-AP antibody diluted 1:3000 in 2% BMB blocking solution, 16.6 µL per 50 mL or individually in 500 uL of antibody solution in 48 well plate wells (Figure 1D)) overnight at 4°C or for 4 hours at room temperature.

23. Wash 5 × 1 hour in MAB at room temperature (or wash overnight at 4°C with multiple quick washes before and after overnight incubation).

24. Wash 2 × 5 minutes in alkaline phosphatase buffer at room temperature.

25. Incubate in BM Purple reagent in wells of a 48 well plate (Figure 1D), protected from light with aluminum foil, and monitor until chromogenic reaction produces a stain of the desired intensity.

    Note: Incubation time in BM Purple varies widely depending on the probe, and can only be determined empirically or by comparison to published literature for a given probe.

    Note: Depending on the stage and tissue interrogated, endogenous pigment may make the visualization of BM Purple precipitate difficult. Bleaching (see below) may make the signal easier to see. Consider this as the chromogenic reaction proceeds.

26. Stop chromogenic reaction with a wash in MAB.

Post-fixation & Bleaching [6 hours]

27. Fix for at least 2 hours at room temperature in Bouin's fixative.

28. Wash 10 × 10 minutes in buffered ethanol solution or until the embryos are no longer yellow.

29. Rehydrate stepwise into 1X SSC at room temperature: 5 minutes 75% buffered ethanol / 25% 1X SSC, 5 minutes 50% buffered ethanol / 50% 1X SSC, 5 minutes 25% buffered ethanol / 75% 1X SSC, 2 × 5 minutes 100% 1X SSC.

30. Bleach in bleaching solution for 1–2 hours at room temperature under a light box or until embryos are white.

31. Wash 3 × 5 minutes in 1X SSC.

32. Samples are ready for imaging.

    Note: Samples can be stored for years at −20°C in methanol or for months at 4°C in 1X SSC.

33. For macro-scale imaging, mount in agarose wells made using 3D printed stamps pressed in molten agarose during cooling (Figure 3).

    Note: 3D printer files for stamps are available at willseyfroggers.org/resources.

34. Alternatively, mount on glass slides in 1X SSC within a vacuum grease well and afix a coverslip.

35. Image using brightfield.

Fluorescence whole-mount RNA *in situ* hybridization by hybridization chain reaction (HCR)

This section describes whole-mount RNA *in situ* hybridization with fluorescent detection by hybridization chain reaction (HCR) (Figure 2B–C) (Choi et al. 2018). This is a more expensive strategy for assaying mRNA expression since it requires commercial RNA probes designed for *Xenopus* sequences (https://www.molecular technologies.org), but provides a great increase in spatial and quantitative resolution over colorimetric detection as well as the ability to label up to 5 RNAs in different wavelengths. Because of the nature of detection, there is also not the objectivity of when to terminate the development of signal, which can be an advantage over colorimetric detection. This method is identical to the previous until prehybridization.

Note: All steps are done on a lateral shaker with light agitation (~40 rpm).

Fixation, Dehydration, Rehydration, Permeabilization, Blocking

2. Carry out steps 1–10 above.

Hybridization [35 minutes until overnight incubation; 1.5 hours until amplification]

3. Prehybridize samples in 30% probe hybridization buffer for 30 minutes at 37°C.

4. Prepare probe solution by adding 2 pmol of each probe mixture (odd & even: 1 μL of 2 μM stock per probe mixture) to 500 μL of 30% probe hybridization buffer at 37°C.

   Note: Probe volume can be reduced to the minimum required to cover samples.

5. Replace the 30% probe hybridization buffer with probe solution and incubate overnight (12–16 hours) at 37°C with shaking.

   Note: For basket format, baskets are removed from the rack and placed into 15 mL round bottom culture tubes (Fisher 1496215E) with 300–500 μL of probe solution each (Figure 1C).

6. Wash 4 × 15 minutes in 30% probe washing solution at 37°C with shaking.

   Note: Save probe solutions. They can be stored at −20°C and reused multiple times.

   Note: Wash solutions should be heated to 37°C before use.

7. Wash samples 3 × 5 minutes in 5X SSCT at room temperature with shaking.

Amplification [35 minutes until overnight incubation; 1.5 hours until mounting]

8. Preamplify samples in amplification buffer for 30 minutes at room temperature.

9. Prepare 30 pmol hairpin solutions in amplification buffer by heating hairpins at 95°C for 90 seconds, cooling to room temperature in a dark drawer for 30 minutes, and then adding to amplification buffer at room temperature. For each sample, 10 μL of each desired 3 μM hairpin is prepared for a 500 μL volume of amplification buffer.

10. Transfer samples into the hairpin solution and incubate overnight (12–16 hours) in the dark at room temperature.

    Note: Hairpin solutions can be stored at −20°C and reused multiple times. For basket format, baskets are removed from the rack and placed into 15 mL round bottom culture tubes (Fisher 1496215E) with 300–500 μL of hairpin solution each (Figure 1C).

11. Wash 2 × 5 minutes, 2 × 30 minutes, and then 1 × 5 minutes in 5X SSCT at room temperature.

12. Wash 3 × 5 minutes in 1X SSC.

13. Samples are ready for imaging.

    Note: Samples can be stored for weeks in the dark at 4°C in 1X SSC.

36. For macro-scale imaging, place in 1X SSC in agarose wells made using 3D printed stamps pressed in molten agarose during cooling (Figure 3). Image on an upright stereomicroscope with fluorescence.

    Note: 3D printer files for stamps are available at willseyfroggers.org/resources.

37. For higher-magnification imaging, mount in 1X SSC in a vacuum grease well on a glass slide, afix coverslip, and image.

Whole-mount immunofluorescence

This section describes whole-mount immunostaining with fluorescent detection (Figure 2D). While it is optimized for tadpole stages, it also works well for many epitopes in earlier stages, including before gastrulation. See Table 1 for a list of primary antibodies compatible with this protocol, particularly for stage 46 tadpoles. Since this is one of the simpler protocols available, this is a good protocol to try first. If no signal is seen, refer to other excellent protocols for additional steps (dehydration, etc), alternative fixatives, and additional positive control antibodies (Lee et al. 2008; Brooks and Wallingford 2015). All incubations (excluding antibody incubations) are done on a lateral shaker (basket format) or on a nutator (tube format).

Fixation [1 hour]

1. Fix in 4% Paraformaldehyde in PBS for 40 minutes at room temperature.

2. Wash in PBS 3 × 5 minutes at room temperature.

Bleaching & Permeabilization [2 hours, 5 minutes]

3. Bleach in bleaching solution for 1 hour at room temperature under a light box.

Note: This step is incompatible with phalloidin staining and will quench any fluorescent proteins (for example, GFP), and should be omitted in those cases.

Note: This step will create bubbles. If using tubes, transfer samples to a glass dish or open tube tops to allow for gas release.

Note: This step will remove pigmentation and provide some permeabilization. This step should not be prolonged as it can begin to disintegrate the sample if carried out for too long.

4. Permeabilize in PBT by washing 3 × 20 minutes at room temperature.

Blocking & Primary Antibody [1 hour until overnight incubation]

5. Block in 10% CAS-Block in PBT for at least 1 hour at room temperature.

6. Incubate in primary antibody diluted in 100% CAS-Block overnight at 4°C.

Note: If using baskets, move baskets into 48 well plates with 300 μl of antibody per well (Figure 1D). If using tubes, volume is minimum to cover embryos completely.

Note: A reasonable starting concentration for a new antibody is 1:100, but should be optimized empirically. For unconcentrated sera (for example, from DSHB), start with 1:5 dilution.

Washes & Secondary Antibody [3 hours, 10 minutes]

7. Wash in PBT 3 × 10 minutes at room temperature.

8. Block in 10% CAS-Block in PBT for 30 minutes at room temperature.

9. Incubate in secondary antibody diluted in 100% CAS-Block for 2 hours in the dark at room temperature.

Note: If using baskets, move baskets into 48 well plates with 300 μl of antibody per well (Figure 1D). If using tubes, volume required is the minimum to cover embryos completely.

Note: If using fluorescence-conjugated secondary antibodies, cover with aluminum foil to protect from the light for the remainder of the staining.

Note: A typical commercial antibody dilution for this step is 1:250. Additional fluorescent dyes can be added during this step (for example, DAPI).

Washes & Mounting [1.5 hours until mounting]

10. Wash 3 × 10 minutes in PBT at room temperature.

11. Wash 3 × 20 minutes in PBS at room temperature.

12. Samples are ready for imaging.

Note: Samples can be stored for a few weeks at 4°C in 1X PBS. If in solution, rather than mounted, add gentamicin (50 μg/ml) to the 1X PBS to extend storage time. A post-fixation can also extend storage time if necessary.

13. For macro-scale imaging, place in 1X PBS in agarose wells made using 3D printed stamps pressed in molten agarose during cooling (Figure 3). Image on an upright stereomicroscope.

14. For higher-magnification imaging, mount in 1X PBS in a vacuum grease well on a glass slide, afix coverslip, and image.

Troubleshooting

Problem: Superficial staining but absence of deeper tissue staining.

Solution: Increase permeabilization by a longer or more concentrated proteinase K treatment (for RNA hybridization), a longer or more concentrated detergent treatment (for RNA or protein staining), or by physically dissecting the tissue to expose the target region.

Problem: Tissue disintegrates during the protocol.

Solution: Increase fixation time and/or decrease proteinase K or detergent washes.

Problem: Weak antibody staining.

Solution: Empirically test alternative fixatives (for example, try glutaraldehyde), antibody concentration, bleaching time, and/or detergent concentration.

Problem: Excessive background staining.

Solution: Increase stringency steps (longer 0.2X SSC washes and increased temperature for RNA hybridization; increased blocking time and permeabilization for immunostaining).

Discussion

This protocol should be modified according to the developmental stage and tissue type of interest. For example, dehydration is often helpful in earlier, more yolky stages, while it can interfere with staining in later tadpole stages. Some tissues and stages require physical permeabilization (for example, removal of skin in later tadpole stages to better permeabilize the brain), while superficial tissues may need less permeabilization (for example, omit the proteinase K step for epidermal cilia staining). Further, some antibodies produce better results with a particular fixative, permeabilization condition, etc., and require empirical testing to optimize. Fluorescence-based detection has the advantage of being able to image in a more quantitative manner at higher spatial resolution. However, HCR probes can be prohibitively expensive, although they can be reused. Finally, the colorimetric RNA *in situ* protocol is derived from a widely-used contribution from Joanna Yeh and Mustafa Khokha according to (Sive et al. 2000) and originally described in (Harland 1991). The fluorescent RNA *in situ* protocol is derived from (Choi et al. 2018) and the imaging stamps are derived from (Truchado-Garcia et al.).

Recipes

Alkaline Phosphatase Buffer

Final Concentration	for 1 L
100 mM Tris (pH 9.5)	100 mL 1 M Tris pH 9.5
50 mM $MgCl_2$	50 mL 1M $MgCl_2$
100 mM NaCl	25 mL 4M NaCl
0.1% Tween 20	1 mL Tween 20
2 mM tetramisole hydrochloride	2 mL 1M tetramisole hydrochloride (Sigma L9756)
	Adjust to 1 L with H_2O and store −20°C in 50 mL aliquots

Amplification Buffer

Final Concentration	for 40 mL
5X SSC	10 mL 20X SSC
0.1% Tween-20	400 µL 10% Tween-20
10% dextran sulfate	8 mL 50% dextran sulfate
	Adjust to 40 mL with H_2O

Bleaching Solution

Final Concentration	for 100 mL
1X PBS (immuno) or 0.5X SSC (ISH)	10 mL 10X PBS + 81 mL H_2O or 2.5 mL 20X SSC + 88.5 mL H2O
5% formamide	5 mL formamide
1.2% peroxide	4 mL of 30% peroxide

Note: Add the formamide and peroxide to the larger volume of PBS or SSC, in that order only. Formamide and peroxide can become explosive if mixed directly alone.

BMB Blocking Solution

Final Concentration	for 500 mL
10% BMB Blocking Agent	50 g BMB Blocking Agent (Sigma 11096176001)
1X MAB	50 mL 10X MAB
	Adjust to 500 mL with H_2O, mix with heat until dissolved
	Store at this 10% concentration at −20°C in 50 mL aliquots
	Dilute to 2% with 1X MAB on staining day

Bouin's Fixative

Final Concentration	for 100 mL
70% saturated picric acid	70 mL saturated picric acid
9.25% formaldehyde	25 mL 37% formaldehyde

Final Concentration	for 100 mL
5% glacial acetic acid	5 mL glacial acetic acid

Buffered Ethanol

Final Concentration	for 4 L
92.625% Ethanol	3.9 L 95% Ethanol
25 mM Tris, pH 8	100 mL 1 M Tris, pH 8
1.25 mM EDTA	10 mL 0.5 M EDTA
	Adjust to 4 L with H_2O

Denhardt's Solution (100 X)

Final Concentration	for 500 mL
2% Ficoll	10 g Ficoll
2% polyvinylpyrrolidone	10 g polyvinylpyrrolidone
2% bovine serum albumin	10 g bovine serum albumin (fraction V)
	Adjust to 500 mL with H_2O
	Filter and store at −20°C in 25 mL aliquots

Dextran Sulfate (50%)

Final Concentration	for 40 mL
50% dextran sulfate	20 g dextran sulfate powder
	Adjust to 40 mL with H_2O

Hybridization Buffer (colorimetric protocol)

Final Concentration	for 3 L
50% formamide	1.5 L formamide
5X SSC	750 mL 20X SSC
1 mg/ml Torula RNA	3 g Torula RNA Type IX (Sigma R3629)
100 μg/mL heparin	0.3 g heparin (Sigma H3393)
1X Denhart's solution	30 mL 100X Denhardt's solution
0.1% Tween 20	3 mL Tween 20
0.1% CHAPS	3 g CHAPS (Sigma C3023)
10 mM EDTA	11.167 g EDTA
	Adjust to 3 L with H_2O, check that pH ~ 7.5, and store −20°C

Hybridization Buffer (30%, fluorescence protocol)

Final Concentration	for 40 mL
30% formamide	12 mL formamide
5X SSC	10 mL 20X SSC
9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
0.1% Tween 20	400 µL 10% Tween 20
50 µg/mL heparin	200 µL 10 mg/mL heparin
1X Denhardt's	400 µL 100X Denhardt's solution
10% dextran sulfate	8 mL 50% dextran sulfate
	Adjust to 40 mL with H_2O, store −20°C

Maleic Acid Buffer (MAB, 10X)

Final Concentration	for 4 L
1 M maleic acid	464.4 g maleic acid (Sigma M0375)
1.5 mM NaCl	350.6 g NaCl
	pH to 7.2 with ~ 280 g NaOH
	Adjust to 4L with H_2O

Note: Adjusting pH here is an exothermic acid-base reaction and often takes a long time. Mix in 4°C room, 70 g of NaOH at a time.

MEMFA (1X)

Final Concentration	for 100 mL
1X MEM salts	10 mL 10X MEM salts
3.7% formaldehyde	10 mL 37% formaldehyde
	80 mL H_2O

MEM Salts (10X)

Final Concentration	for 1 L
1 M MOPS	209.3 g MOPS
20 mM EGTA	7.6 g EGTA
10 mM $MgSO_4$	1.2 g $MgSO_4$
	Adjust to 1 L with H_2O
	pH to 7.4 with NaOH and autoclave 20 minutes.

Note: 10X MEM salts turn yellow after autoclaving.

Paraformaldehyde (20%)

Final Concentration	for 500 mL
20% paraformaldehyde	100 g paraformaldehyde
	500 mL H_2O

Prepare 20% paraformaldehyde by boiling distilled water and then adding 20 g solid paraformaldehyde per 100 ml of water with stirring. Add NaOH pellets until the paraformaldehyde is dissolved. Aliquot in 50 mL tubes and store at −20°C. To make 4% paraformaldehyde fixative solution, thaw 20% aliquots (may need to heat in a water bath for solute to go back into solution) and dilute to 4% in PBS (immunostaining) or PTw (ISH) on staining day.

Phosphate-buffered saline (10X PBS, pH 7.4)

Final Concentration	for 4 L
1.37 M NaCl	320 g NaCl
27 mM KCl	8 g KCl
100 mM Na_2HPO_4	57.6 g Na_2HPO_4
18 mM KH_2PO_4	9.6 g KH_2PO_4
	Adjust to 4 L with H_2O
	pH to 7.4

PBS with Triton X-100 (PBT, 0.1%)

Prepare PBT by adding 0.1% Triton X-100 to 1X PBS (example, add 1 ml Triton X-100 to 1 L of PBS).

PBS with Tween 20 (PTw, 0.1%)

Prepare PTw by adding 0.1% Tween 20 to 1X PBS (example, add 1 ml Tween 20 to 1 L of PBS).

Probe Wash Buffer (30%)

Final Concentration	for 40 mL
30% formamide	12 mL formamide
5X SSC	10 mL 20X SSC
9 mM citric acid (pH 6.0)	360 µL 1 M citric acid, pH 6.0
0.1% Tween 20	400 µL 10% Tween 20
50 µg/mL heparin	200 µL 10 mg/mL heparin
	Adjust to 40 mL with H_2O

Proteinase K Solution

Final Concentration	for 100 mL
10 µg/mL proteinase K	100 µL 10 mg/mL proteinase K (Fisher EO0491)
	100 mL PTw

Sodium Chloride Sodium Citrate Buffer (SSC, 20X)

Final Concentration	for 4 L
3 M NaCl	701.1 g NaCl
0.3 M Na_3citrate • $2H_2O$	352.8 g Na_3citrate • $2H_2O$
	pH to 7.0 with HCl or 10N NaOH
	Adjust to 4 L with H_2O

SSCT (5X)

Final Concentration	for 40 mL
5X SSC	10 mL 20X SSC
0.1% Tween 20	400 µL 10% Tween 20
	Adjust to 40 mL with H_2O

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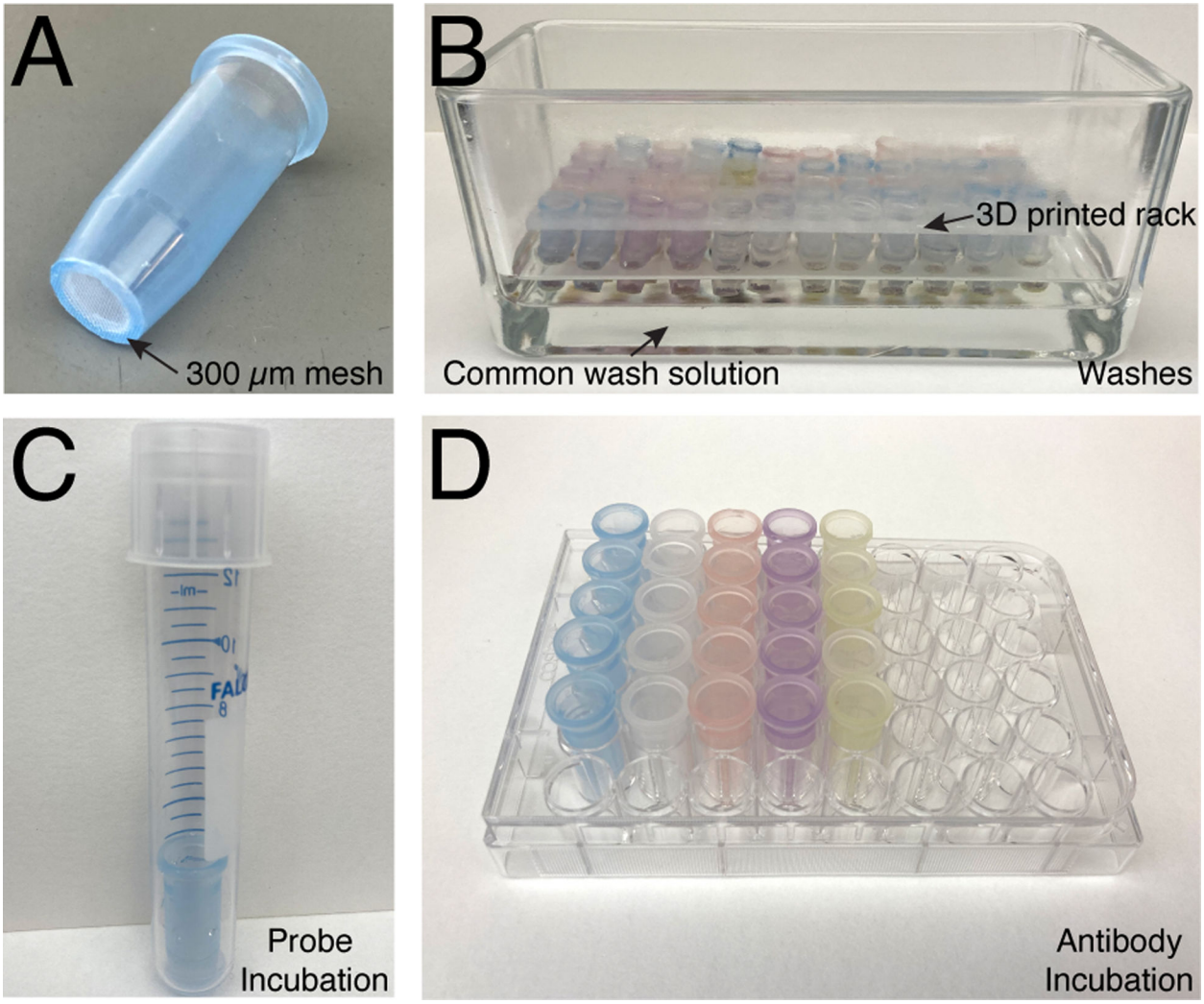

Figure 1. Basket format for higher-throughput staining.

A) Sample basket made from 1.7 mL eppendorf tube (with cap and bottom cut off) with 300 μm mesh melted to the bottom. B) Wash setup, with color-coded baskets arranged in a 3D-printed rack within a glass staining dish. Samples share a common wash buffer. C) Probe incubation setup, in baskets within 15 mL round bottom culture tubes with 500 μL of probe solution. D) Antibody incubation setup within 48 well culture plate with 500 μL of antibody solution each. These incubation setups allow for each sample to experience a different probe or antibody, if desired, and minimize total volume required.

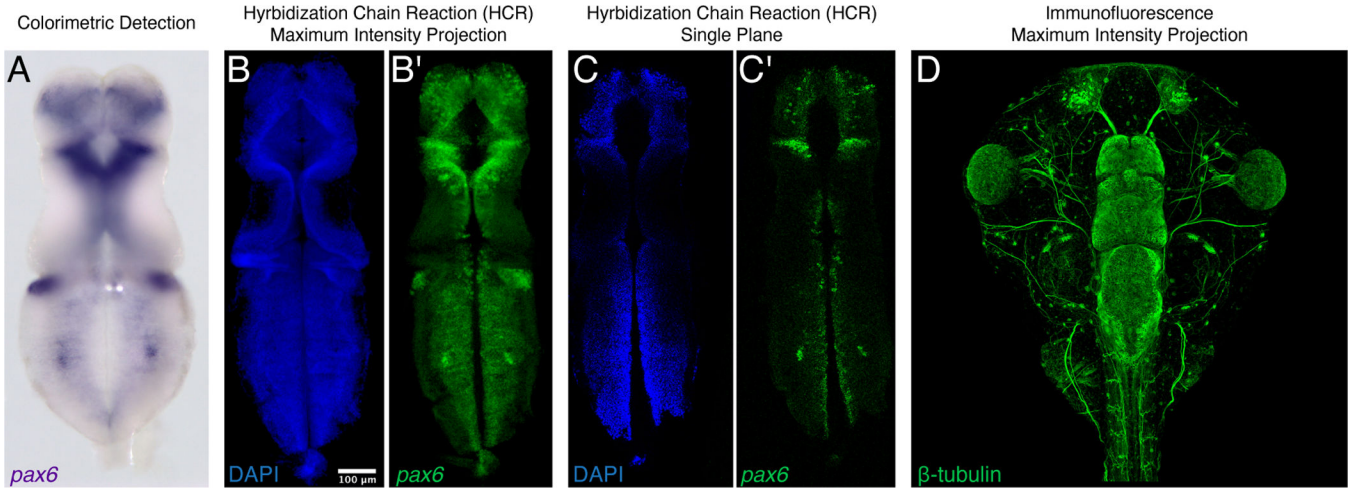

Figure 2. Sample RNA *in situ* hybridization and immunostaining micrographs.

A) Colorimetric staining for *pax6* RNA (purple) in a stage 46 *X. tropicalis* dissected brain imaged by widefield microscopy. B-C) Fluorescence staining for *pax6* RNA by HCR (green, B', C') co-stained with DAPI to label nuclei (blue, B, C) in a stage 46 *X. tropicalis* dissected brain imaged by confocal microscopy. B-B') Maximum intensity projection of confocal sections. C-C') Single imaging plane. Note increased resolution potential with the fluorescence-based method. D) Immunostaining for β-tubulin in stage 46 *X. tropicalis* head region imaged by confocal microscopy.

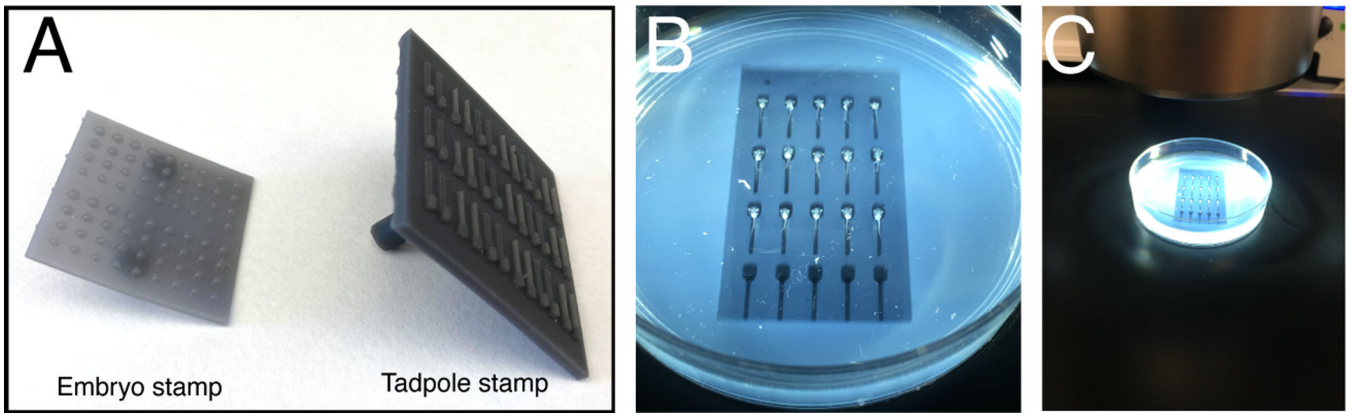

Figure 3. Imaging in agarose wells.

A) 3D-printed stamps for positioning embryos (left) or tadpoles (right). Agarose wells can be made by pressing these into molten agarose in a culture dish during cooling. B) Tadpoles arranged into agarose wells. C) Imaging animals using an upright stereoscope in agarose wells.

Table 1.

Common antibodies compatible with this immunostaining protocol in stage 46 tadpoles along with relevant information.

Antigen	Labels	Host	Company	Product Number	Dilution
β-tubulin	Neurons, microtubules	Mouse	DSHB	E7	1:100
PCNA	Cells in S phase	Mouse	Life Technologies	133900	1:50
pHH3	Cells in M phase	Rabbit	Millipore	06–570	1:250
α-tubulin	Spindles, microtubules	Mouse	DSHB	12G10	1:100
Ac-α-tubulin	Cilia	Mouse	Sigma	T6793	1:700
Cleaved Caspase 3	Cell death	Rabbit	BD pharmigen	559565	1:250