



Chapter 11

Fluorescent In Situ mRNA Hybridization (FISH) Using Hybridization Chain Reaction (HCR) in *Xenopus* Cryosections

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Abstract

Hybridization Chain Reaction (HCR) enables highly sensitive and multiplexed detection of mRNA with subcellular spatial resolution. It employs fluorophore-tagged DNA hairpins that self-assemble on target-bound probe pairs, amplifying the signal without enzymatic reactions. Here, we describe a protocol for performing HCR fluorescence in situ hybridization (FISH) on *Xenopus* tissue sections. We also outline a semi-automated image analysis pipeline that enables per-cell quantification of probe expression and co-localization. This method provides a robust and quantitative approach for visualizing gene expression patterns in situ.

Keywords mRNA FISH, Hybridization chain reaction, In situ hybridization, Fluorescence microscopy

1 Introduction

In situ hybridization (ISH) has been a cornerstone in visualizing spatial gene expression patterns during *Xenopus* development [1–6]. While traditional chromogenic ISH provides robust detection of mRNA, it is limited in sensitivity, quantitative resolution, and multiplexing capability. In contrast, fluorescent in situ hybridization (FISH) using the Hybridization Chain Reaction (HCR) offers an enzyme-free amplification strategy that achieves a high signal-to-noise ratio and enables simultaneous detection of multiple mRNA transcripts (Fig. 1). This approach has proven ideal for fine-scale mapping of cell types, marker co-expression, and pathway activity, thereby providing transcriptomic datasets with an in situ spatial context (Figs. 2 and 3) [7–11].

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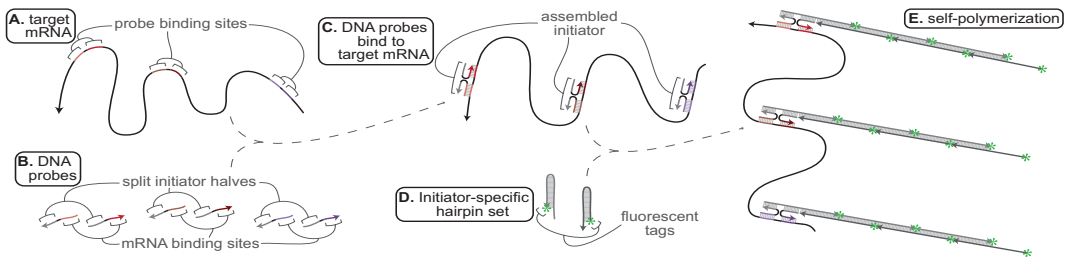


Fig. 1 Schematic representation of in situ HCR v3.0 using split-initiator probes. **(A)** Target mRNA with neighbouring sets of adjacent pairs of binding sites for a pool of DNA probes. **(B)** DNA probe pairs with highlighted regions for binding onto neighboring sites of the target mRNA, and one half of the hairpin initiator sequence. **(C)** The binding of each initiator probe pair to adjacent sequences on the target mRNA colocalizes the initiator halves. **(D)** Complementary metastable hairpins carrying a fluorescent tag. **(E)** Assembled initiators drive the hairpins to bind onto them, thus revealing the binding domain for their sister hairpin and leading to the polymerization chain reaction that amplifies the signal. (Based on Choi et al. [7])

HCR employs fluorophore-labeled DNA hairpins that self-assemble into polymeric chains when triggered by an initiator sequence hybridized to a targeted RNA through a specific probe pair (Fig. 1). The latest version of the technique, HCR v3.0, introduced by Choi et al. [7], makes use of split-initiator probes—oligonucleotide probe pairs that bind to adjacent sequences within the target mRNA, each carrying one half of the initiator sequence recognized by the hairpins (Fig. 1). Polymerization is therefore initiated only when both initiator halves are brought together on the target, resulting in high signal amplification with minimal background [7].

HCR can be performed in both whole-mount and sectioned tissue. It preserves the morphological structure of the specimen, supports multiplex detection of up to ten different mRNAs, and is compatible with immunohistochemistry and dye labeling (Figs. 2 and 3) [7, 12–14]. The versatility of HCR makes it well-suited to amphibian research, particularly in *Xenopus laevis*, a model system with powerful developmental genetics but a more limited antibody and transgenic toolkit compared to zebrafish or mice. The ability to design probes for virtually any *Xenopus* transcript, combined with compatibility with standard fixation and robustness across developmental stages, makes HCR a valuable addition to the *Xenopus* molecular toolbox.

This chapter provides a detailed protocol for performing HCR-FISH in *Xenopus laevis* cryosections, including tissue preparation, labeling, imaging, and quantitative analysis. Notes on optimization, multiplexing, and troubleshooting are provided to facilitate the application of HCR across developmental stages and tissue types.

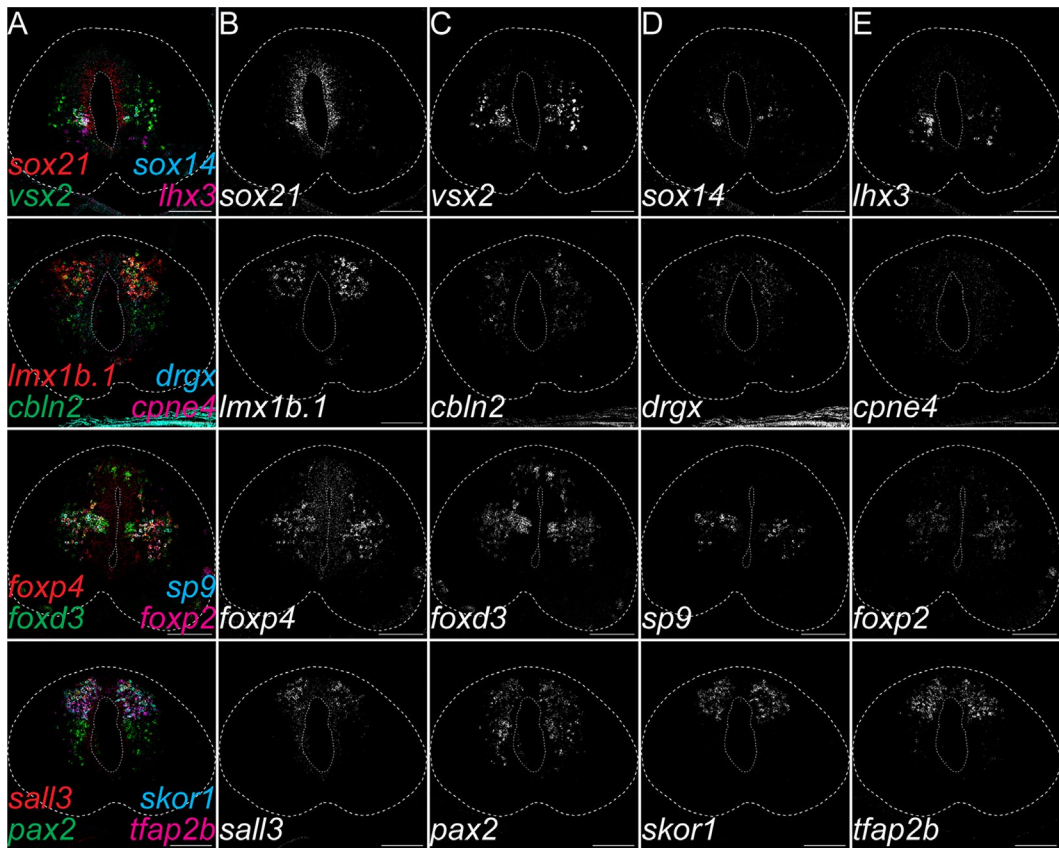


Fig. 2 HCR multiplexing in the *Xenopus laevis* spinal cord. **(A)** Four multiplex HCR combinations, each labeling four neuronal markers, in transverse spinal cord sections of stage NF-55 *Xenopus laevis* [16]. **(B–E)** The individual markers depicted in **(A)** as labeled. Scale bars, 100 μm

2 Materials

2.1 Reagents and Buffers

All reagents should be RNase-free, and water should be DEPC-treated.

1. Phosphate-buffered saline (PBS): 1×, without CaCl_2 or MgCl_2 .
2. 4% Paraformaldehyde (PFA) in PBS.
3. Ethanol (EtOH): absolute ethanol ($\geq 99.8\%$), prepared as 50%, 70%, and 100% (v/v) solutions for dehydration steps.
4. 20 mg/mL proteinase K stock; dilute to 10 μg/mL in PBST.
5. Probe Hybridization Buffer (Molecular Instruments) (*see Note 1*).
6. Probe Wash Buffer (Molecular Instruments) (*see Note 1*).
7. Amplification Buffer (Molecular Instruments) (*see Note 1*).

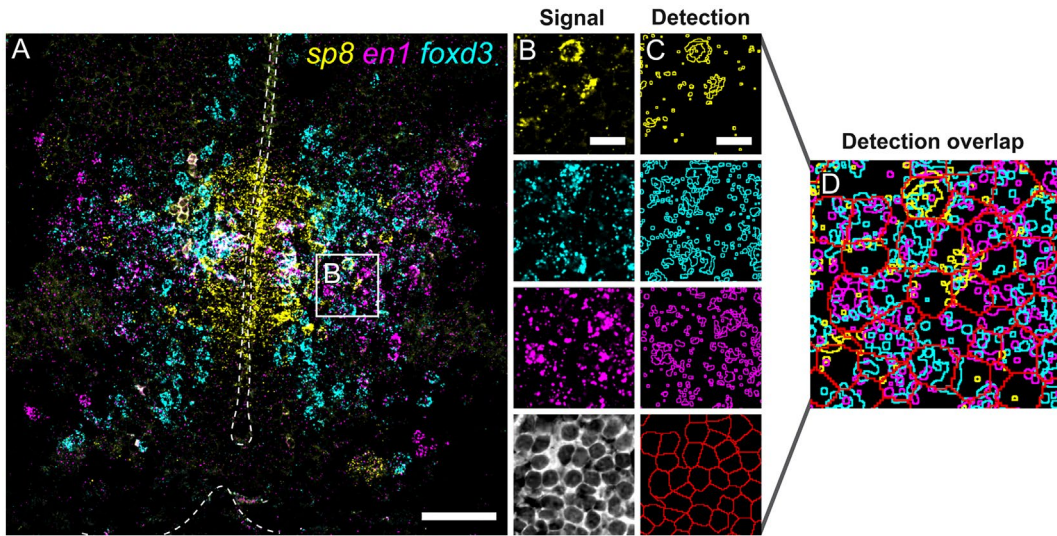


Fig. 3 HCR speckle detection and per-cell quantification pipeline. **(A)** Representative transverse spinal cord section of *Xenopus laevis* showing multiplexed HCR signal for sp8 (yellow), en1 (magenta), and foxd3 (cyan). The dashed line indicates the central canal; the boxed region indicates the area shown in panels B–D. **(B)** Zoomed view of individual HCR signal channels (top to bottom: sp8, en1, foxd3) and cell boundary channel (CellBrite® Blue labeling) used for cell segmentation. **(C)** Corresponding detection masks generated by QuPath pixel classifiers for each HCR channel and Cellpose-based cell segmentation (red outlines). **(D)** Overlay of detected HCR speckles assigned to individual segmented cells, illustrating per-cell signal attribution used for downstream co-expression analysis. Scale bars: **(A)** 40 μm ; **(B–D)** 10 μm

8. 5 $\times$ SSCT: 5 $\times$ Sodium chloride-sodium citrate (SSC) containing 0.1% Tween-20. For 5 $\times$ SSC, dilute 20 $\times$ SSC [0.3 M sodium citrate, 3 M sodium chloride, pH 7.0] 1:4 with MilliQ H₂O.
9. Split-initiator probe sets (B1–B5 series) targeting transcripts of interest, 1 μM stocks (Molecular Instruments).
10. HCR hairpins (H1 and H2 for each initiator), 3 μM stocks (Molecular Instruments).
11. Mounting medium: PV-DABCO or Mowiol antifade reagent.
12. 10 $\times$ MMR+ (Marc's Modified Ringer's with EDTA): 58.4 g NaCl, 1.49 g KCl, 2.46 g MgSO₄·7H₂O, 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 CaCl₂·2H₂O. Dilution to 0.1 $\times$ results in a final pH of ~7.4.
13. 0.01% (w/v) MS-222 anesthetic: 0.1 g ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma, #E10521), 0.15 g NaHCO₃ in 1000 mL 0.1 $\times$ MMR+.
14. 50% (w/v) fish jelly: 10 g fish gelatin in 10 mL MilliQ H₂O, dissolved at 37 $^{\circ}\text{C}$; adjust to 20 mL with MilliQ H₂O. Store at 4 $^{\circ}\text{C}$.

15. Fish jelly-sucrose cryoprotectant: 7.5 g sucrose in 25 mL 1× PBS supplemented with 4 g room temperature fish jelly; adjust to 50 mL with 1× PBS (*see Note 2*).
16. Isopentane (2-methylbutane) (Sigma, #M32631).
17. Dry ice (for chilling isopentane).
18. Optional: Cell membrane dye (e.g., CellBrite® Blue; Biotium, #30024) for cell boundary visualization (during image analysis).

2.2 Equipment

1. Hybridization oven or laboratory incubator set to 37°C.
2. Humidified hybridization chamber (e.g., slide box with wet paper towels).
3. Water bath (set to 37°C).
4. Heat block (set to 95°C).
5. RNase-free pipette tips.
6. Fine forceps.
7. Slide boxes for fixation and dehydration steps.
8. Parafilm sheets cut to slide size (used as coverslips during hybridization and amplification). Cut a Parafilm sheet into rectangles that cover the area of the slide occupied by tissue. Prepare three Parafilm coverslips per slide (used for the following incubation steps: proteinase K, hybridization, and amplification).
9. Tissue embedding molds: TT Cryomold® Standard, square (25×20×5 mm) (Tissue-Tek, #4557).
10. Tissue freezing medium (cryo-embedding medium; e.g., Electron Microscopy Sciences, #72592).
11. Lint-free wipes (such as Kimwipes).
12. 1.5 mL microcentrifuge tubes, nuclease-free.

3 Methods

3.1 Tissue Preparation

1. Animals are euthanized in 0.01% MS-222 in 0.1× MMR+ (*see Note 3 and 4*).
2. Fix tissue in 4% PFA for 1.5 h on ice (*see Note 5*).
3. Wash tissue three times with 4°C 1× PBS.
4. Transfer tissue into jelly-sucrose cryoprotectant solution, incubate overnight on a shaker at 4°C (*see Note 6*).
5. Fill a vinyl mold with tissue freezing medium.
6. Dry tissue by gently dabbing on a lint-free tissue.
7. Gently immerse the tissue in a vinyl mold filled with tissue freezing medium (*see Note 7*).

8. Snap-freeze samples by partially submerging them in a glass container filled with isopentane kept on dry ice (*see* **Note 8**).
9. Store samples at -80°C until sectioning.

3.2 Probe Preparation

Probes can either be ordered as custom-designed sets directly from Molecular Instruments®, or designed in-house based on the *X. laevis* genome (Xenbase; RRID:SCR_003280; *see* **Note 9**) [15] and synthesized by commercial oligonucleotide providers (e.g., Integrated DNA Technologies; RRID:SCR_025813). Probe pools containing fewer than 10–12 probe pairs typically yield weak or undetectable signal. Once above 10–12 pairs, increasing the number of probe pairs does not substantially improve labeling efficiency; instead, signal intensity can be adjusted by modifying probe concentration (*see* **Probe Hybridization** section, and **Note 19**). When designing probe pools for multiplexing, select hairpin fluorophores with minimally overlapping excitation and emission spectra to avoid the need for spectral unmixing during imaging (*see* **Note 10**). For access to our custom *X. laevis*-specific probe design pipeline and accompanying code, please refer to the following ISTA GitLab page: https://git.ista.ac.at/research-software/hcr_probe.

3.3 HCR Labeling of Tissue Cryosections

Adapted from the Molecular Instruments protocol “HCR v3.0: Fresh Fixed Frozen Tissue” (version 2021.03.12)

Day 0—Section Preparation

Dry sections (stored at -80°C or freshly cut) by placing slides at 4°C overnight, or 30 min at room temperature on a metallic surface, followed by 5–6 h at 4°C , always protected from dust.

Day 1—Sample Preparation (~2 h)

1. Thaw 4% PFA at room temperature and place on ice.
2. Fix sections by immersing slides into cold 4% PFA for 15 min at 4°C (*see* **Note 11**).
3. Pre-warm a humidified chamber in an oven at 37°C (*see* **Note 12**).
4. Remove slides from the PFA and drain the excess.
5. Wash slides sequentially in 50%, 70%, and two changes of 100% ethanol (5 min each, room temperature) to dehydrate tissue (*see* **Note 13**).
6. Air-dry slides for 5 min at room temperature

Proteinase K treatment (~15 min) (Optional, *see* **Note 14**).

7. Thaw proteinase K solution (from -20°C). (Thawing time ~10–15 min.)
8. Place the slides in the pre-warmed, humidified chamber. Add 200–300 μL of 10 $\mu\text{g}/\text{mL}$ proteinase K solution onto each slide (*see* **Note 15**).

9. Cover with a Parafilm slip and incubate for 10 min at 37°C.
10. Remove Parafilm carefully and wash twice in 1× PBS (*see* **Notes 16–18**).

Day 1—Probe Hybridization (~15 min + overnight incubation)
Start **Steps 1 and 2** while the incubations of the previous steps are running.

1. Set a water bath to 37°C.
2. Prepare 1.5 mL microcentrifuge tubes with probe combination solution by adding 0.4 µL of 1 µM probe stock (0.4 pmol) of each required probe set to 100 µL of Probe Hybridization Buffer and warm them in a 37°C water bath (*see* **Note 19**).
3. Drain the previous solution and blot the slide edges on a lint-free tissue.
4. Cover each slide with 200–330 µL of Probe Hybridization Buffer and incubate in a humidified chamber for 10 min at 37°C (*see* **Note 20**).
5. Drain buffer and blot slide edges again.
6. Add 50–100 µL of the pre-warmed probe solution to each slide and cover with Parafilm. Incubate overnight (12–16 h) in a humidified chamber at 37°C.

Day 2—Detection stage (~2 h)

The slides are incubating in a humidified chamber at 37°C.

1. Set a water bath to 37°C.
2. Warm 200–350 µL of Probe Wash Buffer per slide in a 37°C water bath.
3. Warm 200–350 µL of each of the four solutions needed for Step 7 to 37°C in a water bath.
4. Carefully remove the Parafilm from each slide and cover the tissue with 200–350 µL of pre-warmed Probe Wash Buffer.
5. Turn on a heat block and set it to 95°C for hairpin preparation (*see* **Hairpin Preparation** section).
6. Set aside 200–300 µL of Amplification Buffer per slide at room temperature to equilibrate (*see* **Note 21**).

Important: Perform **Steps 7–9** in parallel with **Hairpin Preparation, Steps 10–14**.

7. Drain the previous solution, and wash slides sequentially for 15 min each at 37°C in a humidified chamber using pre-warmed washes as follows: (i) 200–350 µL of 75% Probe Wash Buffer/25% 5× SSCT, (ii) 200–350 µL of 50% Probe Wash Buffer/50% 5× SSCT, (iii) 200–350 µL of 25% Probe Wash Buffer/75% 5× SSCT, (iv) 200–350 µL of 100% 5× SSCT.

8. Prepare a new humidified chamber for incubation at room temperature.
9. Transfer slides to the new chamber and wash for 5 min at room temperature in 200–350 μ L of 100% 5 $\times$ SSCT.

Hairpin Preparation (begin at the same time as **Step 7** of the **Detection stage**)

10. Thaw the required hairpin pairs (H1 and H2) and place them on ice. (Thawing time ~5–10 min.)
11. Prepare individual tubes containing 2 μ L of 3 μ M (6 pmol) hairpin H1 and hairpin H2 per slide (*see Note 22*).
12. Heat hairpins at 95°C for 90 s in a heat block (no mixing required), keeping tubes covered with aluminum foil to protect from light.
13. Snap-cool hairpins by placing tubes in the dark at room temperature for at least 30 min.

Day 2—Amplification Stage (≥ 30 min setup + overnight incubation)

14. Drain the previous solution and cover each slide with 200–300 μ L of room temperature Amplification Buffer. Incubate for 30 min in a humidified chamber at room temperature. (*During incubation, proceed with Steps 15 and 16*) (*see Note 23*).
15. Set aside 100 μ L of Amplification Buffer per slide at room temperature for hairpin mixing (Step 16).
16. Prepare the hairpin solution for each slide by adding snap-cooled H1 and H2 hairpins to 100 μ L of room temperature Amplification Buffer (*see Notes 24 and 25*).
17. Drain the previous solution and gently blot the slide edges on a lint-free wipe.
18. Add 100 μ L of the prepared hairpin solution onto each slide, ensuring complete tissue coverage. Cover each slide with a Parafilm slip to prevent evaporation.
19. Incubate slides overnight (12–16 h) in a dark, humidified chamber at room temperature.

Day 3—Amplification Stage (≥ 1 h 35 min)

1. Remove the Parafilm carefully and wash slides sequentially at room temperature in a dark, humidified chamber with 350–400 μ L of 100% 5 $\times$ SSCT, for 2 $\times$ 30 min and 1 $\times$ 5 min. At this point, one can proceed with antibody staining and/or dye labeling if desired (*see Note 26*).
2. Drain the final wash and gently blot the slide edges on a lint-free tissue.
3. Mount a glass coverslip using 80–100 μ L of PV-DABCO mounting medium.

4. Keep slides in the dark at room temperature for at least 1 h to allow the mountant to set.
5. Clean the underside of the slides with 100% ethanol.
6. Store the slides at 4°C in the dark.

3.4 HCR Per-Cell Quantification

Quantifying HCR-FISH signal at single-cell resolution enables assessment of gene expression and co-expression patterns across tissue regions, experimental conditions, and phenotypes. Here, we describe a semi-automated analysis pipeline that integrates Fiji, QuPath, and CellPose to segment cells, detect HCR puncta, and extract per-cell fluorescence measurements. A user-friendly interface implemented in Marimo (Python 3.12) guides the user from the raw image data to final figures and tabular outputs, enabling reproducible quantification of mRNA presence and co-expression from multiplexed HCR datasets.

All code and the complete analysis pipeline are available on ISTA GitLab: <https://git.ista.ac.at/iof-group/image-analysis/HCR-SpeckleQuantification>

3.4.1 Image Acquisition

Capture full optical z-stacks with a 40× or higher magnification confocal objective. Set z-steps to the Nyquist value for optimal 3D reconstruction and analysis.

3.4.2 Pipeline Setup

For detailed installation instructions, refer to the repository documentation. Briefly, to use the pipeline:

1. Install Fiji, QuPath, and Cellpose.
2. Download or clone the *HCR-SpeckleQuantification* repository.
3. Create and activate the provided virtual environment to launch the Marimo interface.
4. Follow the step-by-step instructions in the Marimo interface.

Important: HCR speckle detection relies on QuPath pixel classifiers, which must be adapted to the user data. Example classifiers are provided with the repository and can be modified as needed (*HCR-SpeckleQuantification/analysis/02_marimo_gui/source-classifiers*).

3.4.3 Image Preprocessing

1. The pipeline analyzes HCR signal across user-defined experimental conditions (e.g., treatment/genotype, experiment, animal).
2. Based on these annotations, a standardized project folder structure is generated automatically, and users assign an appropriate QuPath pixel classifier for each condition.
3. A semi-automated preprocessing step in Fiji, launched through the same interface, prepares raw image data by (i) re-ordering

channels to ensure consistent channel identity, (ii) defining regions of interest (ROIs) (e.g., left/right sides), and (iii) splitting z-stacks into individual slices for import into QuPath.

4. Following preprocessing, one QuPath project per dataset is created automatically, containing prepared images and associated classifiers.

3.4.4 Cell Segmentation

1. Cell segmentation is performed within the user-defined ROIs for all images in each QuPath project. Segmentation is applied to Channel 4, which contains cell boundary signal (see Fig. 3).
2. By default, segmentation relies on the Cellpose cyto3 pre-trained mode, accessed via the QuPath Cellpose extension. Segmentation results can be visually inspected directly in QuPath.

3.4.5 HCR Signal Detection

1. Using the pre-trained pixel classifiers, HCR signal speckles are detected in the relevant fluorescence channels (Channel 1–3 by default) and are assigned to individual segmented cells (see Fig. 3).
2. After completion of QuPath-based analysis, tabular outputs containing measurements for all segmented cells and detected speckles are exported to the output directory.
3. Users may then select a subset of z-slices of interest for each dataset to exclude out-of-focus planes.

3.4.6 Per-Cell Quantification and Co-Expression Analysis

1. In the final analysis step, users define channel names and per-cell area coverage thresholds for co-expression via the interface. Area coverage represents the percentage of a cell's area occupied by speckles in each channel.
2. The pipeline computes per-cell area coverage percentages for each transcript. Cells are classified as positive or negative for a given marker based on user-defined thresholds.
3. Summary figures and tables containing cell positivity and co-expression counts are exported automatically.

4 Notes

1. Probe Hybridization Buffer, Probe Wash Buffer, and Amplification Buffer are commercially available as part of the HCR v3.0 kit (Molecular Instruments); in-house preparation of similar buffers has also been described previously [7].
2. Allow sucrose to fully dissolve before adding fish jelly. Prepare fresh each time.

3. This protocol has been optimized for tadpole tissue spanning the metamorphosis stages (NF42-65 [16]).
4. A detailed protocol for euthanasia at different developmental stages can be found in Chap. 1 of the current book.
5. Tadpoles younger than NF49 [16] are best fixed whole. For older animals dissecting the region of interest before fixation is advised.
6. The tissue can be further trimmed while in the Jelly-Sucrose cryoprotectant solution at this stage.
7. A pair of forceps or a pipette tip can be used. To avoid tissue deterioration and the formation of bubbles, move the tissue as little as possible within the viscous Tissue Freezing Medium.
8. Do not let the Tissue Freezing Medium come in direct contact with isopentane.
9. A detailed guide on how to best use Xenbase can be found in Chap. 3 of the current book.
10. Table 1 shows the optimal combinations based on the excitation and emission spectra of the five common fluorophores 405, 488, 546, 594, and 647: a. two colors, b. & c. three colors, and d. four colors that should be compatible with the specifications of most confocal microscopes and do not require spectral unmixing (Table 1).
11. A 5-slide vertical box can be used, and 18–25 ml of PFA is sufficient for 5 to 1 slides, accordingly. The lid of the slide box should be secured closed with Parafilm.
12. The oven temperature will drop when working with the oven door open. Do not raise the oven temperature, but aim to work swiftly.
13. When no Parafilm slip is used to cover the slide, make sure the sections are completely covered under a convex bubble of liquid so as not to dry out.

Table 1

Optimal combinations based on the excitation and emission spectra of the five common fluorophores 405, 488, 546, 594, and 647: a. two colors, b. & c. three colors, and d. four colors

Wavelength (nm)	405	488	546
488	a, b, c, d		
546	a, d	a	
594	a, b	a	
647	a, c, d	a	a

14. The proteinase K digestion can often be omitted without any impairment of signal. Furthermore, proteinase K can disrupt nuclear morphology, resulting in diffused DAPI and enlarged nuclear staining.
15. To ensure full tissue coverage create a single pool of solution at one end of the slide spanning its whole width and gently lower a slightly bent piece of Parafilm, with the convex side facing the slide, starting at the slide edge with the liquid pool, allowing the solution to slowly spread between Parafilm and slide over the rest of the tissue. (Optional: make a small fold at a corner such that it points up for easy removal.) Alternatively, pipette small drops of the solution at the center and each corner of the slide before lowering the Parafilm slip.
16. Grab one corner of the Parafilm piece with forceps, lift it slightly off the slide, and peel it slowly towards the diagonally opposite corner. Avoid creating shearing forces so as not to disturb the tissue.
17. When removing Parafilm coverslips, work quickly to keep the tissue moist at all times. Even brief drying can increase background signal.
18. Slides may be stored in 1× PBS in a humidified chamber for up to 1 h at 37°C.
19. Probe concentration may be increased, up to two or three times, to enhance signal intensity.
20. When warming solutions, always prepare a few extra microliters to compensate for pipetting and evaporation loss.
21. Amplification Buffer is very viscous; pipette slowly and avoid bubbles.
22. Hairpin pairs H1 and H2 should be prepared separately from each other and from the other hairpin B-types, each aliquoted into its own tube. If a B-type is being used in multiple slides with the same number of B-types, then for this B-type multiples of H1 can be prepared together, as can multiples of H2 (*see Note 25*).
23. This step can be slightly prolonged if needed.
24. Mix the solution just before you are ready to proceed; do not let the solution sit after mixing.
25. For more than one B-type per slide, to end up with only $V_f = 100\ \mu\text{l}$ of solution per slide and the same concentration for all hairpins with the minimum waste of materials, calculate volumes beforehand as described below. (Preparation time $\leq 15\ \text{min}$)

Hairpin solution calculation for multiplexing:

All slides with the same number of B-types, volume of Amplification Buffer per hairpin per B-type:

1. $V_{AB \text{ B-type}} = V_f \cdot N_{\text{of slides with B-type}} / N_{\text{of B-types per slide}}$
2. Add $V_{AB \text{ B-type}}$ in either of the snap-cooled tubes of a hairpin pair (H1 or H2).
3. Mix a few times by pipetting up and down.
4. Transfer $V_{AB \text{ B-type}}$ with the hairpin into its pair's tube, and mix again.
5. Create a tube for each hairpin combination ($T_{\text{of combination}}$).
6. Transfer $V_{AB \text{ B-type}} \cdot N_{\text{of slides with same combination}} / N_{\text{of slides with B-type}}$ to each $T_{\text{of combination}}$ that includes this B-type.

After you have done this for each B-type, each $T_{\text{of combination}}$ should have $V_f = 100 \mu\text{L}$ of the right combination of B-type hairpins, with both H1 and H2 varieties, at the same concentration, for each slide with this hairpin combination.

Example:

Slide 1: B1-target1, B2-target2, B5-target3

Slide 2: B1-target4, B4-target5, B3-target6

Slide 3: B1-target1, B2-target4, B3-target3

Slide 4: B1-target3, B2-target1, B3-target6 (different targets

but same hairpin combination as Slide 3)

Slide 5: B1-target5, B2-target2, B5-target3 (different targets but same hairpin combination as Slide 1)

$$V_{AB \text{ B-type}} = V_f \cdot N_{\text{of slides with B-type}} / N_{\text{of B-types per slide}} \Rightarrow V_{AB \text{ B1}} = 100 \cdot 5 / 3 \Rightarrow V_{AB \text{ B1}} \sim 167 \mu\text{L}$$

$$[\dots] \Rightarrow V_{AB \text{ B2}} = 100 \cdot 4 / 3 \Rightarrow V_{AB \text{ B2}} \sim 133 \mu\text{L}$$

$$[\dots] \Rightarrow V_{AB \text{ B3}} = 100 \cdot 3 / 3 \Rightarrow V_{AB \text{ B3}} = 100 \mu\text{L}$$

$$[\dots] \Rightarrow V_{AB \text{ B4}} = 100 \cdot 1 / 3 \Rightarrow V_{AB \text{ B4}} \sim 33 \mu\text{L}$$

$$[\dots] \Rightarrow V_{AB \text{ B5}} = 100 \cdot 2 / 3 \Rightarrow V_{AB \text{ B5}} \sim 67 \mu\text{L}$$

$$T_{\text{of slide}}: V_{AB \text{ B-type}} \cdot N_{\text{of slides with same combination}} / N_{\text{of slides with B-type}} \Rightarrow$$

$$T_1: V_{AB \text{ B1}} \cdot 2 / 5 + V_{AB \text{ B2}} \cdot 2 / 4 + V_{AB \text{ B5}} \cdot 2 / 2 = 167 \cdot 2 / 5 + 133 \cdot 2 / 4 + 67 \cdot 2 / 2 \sim 66.6 + 66.6 + 66.6 \sim 200 \mu\text{L} (V_f = 100 \mu\text{L for Slide 1 and } V_f = 100 \mu\text{L for Slide 5})$$

$$T_2: V_{AB \text{ B1}} \cdot 1 / 5 + V_{AB \text{ B4}} \cdot 1 / 1 + V_{AB \text{ B3}} \cdot 1 / 3 = 167 / 5 + 33 + 100 / 3 \sim 33.3 + 33.3 + 33.3 \sim 100 \mu\text{L } V_f \text{ for Slide 2}$$

$$T_3: V_{AB \text{ B1}} \cdot 2 / 5 + V_{AB \text{ B2}} \cdot 2 / 4 + V_{AB \text{ B3}} \cdot 2 / 3 = 167 \cdot 2 / 5 + 133 \cdot 2 / 4 + 100 \cdot 2 / 3 \sim 66.6 + 66.6 + 66.6 \sim 200 \mu\text{L} (V_f = 100 \mu\text{L for Slide 3 and } V_f = 100 \mu\text{L for Slide 4})$$

26. To facilitate cell segmentation and per-cell quantification, a lipophilic membrane dye can be applied before mounting. Commercial options such as the CellBrite® series (Biotium) are compatible with HCR labeling and come in multiple emission spectra. Choose a dye color that does not overlap with your HCR fluorophores; for instance, if probes are labeled with Alexa Fluor 488, 546, and 647, a blue-range dye can be added (e.g., #30024 Biotium). This provides clear cell boundary visualization (see Fig. 3) and improves cell segmentation

and downstream quantification. Dyes are typically applied per manufacturer's instructions: 1:100–1:1000 dilution in 1×PBS for 10 min at room temperature, followed by rinsing with 1×PBS.

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References

1. Cho KWY, Blumberg B, Steinbeisser H, De Robertis EM (1991) Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene goosecoid. *Cell* 67:1111–1120. [https://doi.org/10.1016/0092-8674\(91\)90288-A](https://doi.org/10.1016/0092-8674(91)90288-A)
2. Smith WC, Harland RM (1992) Expression cloning of *noggin*, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* 70:829–840. [https://doi.org/10.1016/0092-8674\(92\)90316-5](https://doi.org/10.1016/0092-8674(92)90316-5)
3. Hirsch N, Harris WA (1997) *Xenopus* Pax-6 and retinal development. *J Neurobiol* 32:45–61. [https://doi.org/10.1002/\(SICI\)1097-4695\(199701\)32:1%253C45::AID-NEU5%253E3.0.CO;2-E](https://doi.org/10.1002/(SICI)1097-4695(199701)32:1%253C45::AID-NEU5%253E3.0.CO;2-E)
4. Khokha MK, Chung C, Bustamante EL et al (2002) Techniques and probes for the study of *Xenopus tropicalis* development. *Dev Dyn* 225:499–510. <https://doi.org/10.1002/dvdy.10184>
5. Borday C, Parain K, Tran HT et al (2018) An atlas of Wnt activity during embryogenesis in *Xenopus tropicalis*. *PLoS One* 13:e0193606. <https://doi.org/10.1371/journal.pone.0193606>
6. Gandin V, Kim J, Yang L-Z et al (2025) Deep-tissue transcriptomics and subcellular imaging at high spatial resolution. *Science* 388:eadq2084. <https://doi.org/10.1126/science.adq2084>
7. Choi HMT, Schwarzkopf M, Fornace ME et al (2018) Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development* 145:dev165753. <https://doi.org/10.1242/dev.165753>
8. Shainer I, Kuehn E, Laurell E et al (2023) A single-cell resolution gene expression atlas of the larval zebrafish brain. *Sci Adv* 9:eade9909. <https://doi.org/10.1126/sciadv.ade9909>
9. Woych J, Ortega Gurrola A, Deryckere A et al (2022) Cell-type profiling in salamanders identifies innovations in vertebrate forebrain evolution. *Science* 377:eabp9186. <https://doi.org/10.1126/science.abp9186>
10. Vijatovic D, Toma FA, Ignatyev Y, Harrington ZPM, Sommer C, Hauschild R, Smits M, Dalla Vecchia M, Trevisan AJ, Chapman P, Julseth MJ, Brenner-Morton S, Gabitto MI, Dasen JS, Bikoff JB, Sweeney LB (2026) Multifold increase in spinal inhibitory cell types with emergence of limb movement. *Cell Reports* 45:117227. <https://doi.org/10.1016/j.celrep.2026.117227>
11. Ignatyev Y, Papadopoulos S, Soretić M et al (2025) Innovations in spinal cord cell type heterogeneity across vertebrate evolution. *bioRxiv* 2025.10.09.680955
12. Schwarzkopf M, Liu MC, Schulte SJ et al (2021) Hybridization chain reaction enables a unified approach to multiplexed, quantitative, high-resolution immunohistochemistry and in situ hybridization. *Development* 148(22):dev199847. <https://doi.org/10.1242/dev.199847>
13. André M, Dinvaux S, Castellani V, Falk J (2024) 3D exploration of gene expression in chicken embryos through combined RNA fluorescence in situ hybridization, immunofluorescence, and clearing. *BMC Biol* 22: 131. <https://doi.org/10.1186/s12915-024-01922-0>

14. Schulte SJ, Fornace ME, Hall JK et al (2024) HCR spectral imaging: 10-plex, quantitative, high-resolution RNA and protein imaging in highly autofluorescent samples. *Development* 151:dev202307. <https://doi.org/10.1242/dev.202307>
15. Fisher M, James-Zorn C, Ponferrada V et al (2023) Xenbase: key features and resources of the *Xenopus* model organism knowledgebase. *Genetics* 224:iyad018. <https://doi.org/10.1093/genetics/iyad018>
16. Nieuwkoop P, Faber J (1956) Normal table of *Xenopus laevis* (Daudin). A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis, 1st edn. North-Holland Publishing Company, Amsterdam

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