HCR RNA-FISH v3.0 protocol for whole-mount zebrafish larvae (*Danio rerio*)

This protocol has been optimized for embryos at 27 hpf and larvae at 5 dpf. Other developmental stages may require additional optimization.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Collect zebrafish embryos and incubate at 28 °C in a petri dish with egg H_2O.
2. Exchange egg H_2O with egg H_2O containing 0.003% of 1-phenyl 2-thiourea (PTU) when embryos reach 12 hpf.
   NOTE: *PTU inhibits melanogenesis but can be toxic at high concentrations. PTU treatment must start before the initial pigmentation occurs as PTU does not remove pigment that has already formed. PTU treatment is not necessary for 27 hpf (hours post-fertilization) embryos.*
3. Replace with fresh egg H_2O containing 0.003% of PTU everyday until the larvae reach 5 dpf (days post-fertilization).
4. Transfer ∼40 larvae (5 dpf) to a 2 mL eppendorf tube and remove excess egg H_2O.
5. Fix larvae in 2 mL of 4% paraformaldehyde (PFA) for 24 h at 4 °C.
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
   NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
6. Wash larvae 3 × 5 min with 1 mL of 1× phosphate-buffered saline (PBS) to stop the fixation.
   NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*
7. Dehydrate and permeabilize with a series of methanol (MeOH) washes (1 mL each):
   (a) 100% MeOH for 4 × 10 min
   (b) 100% MeOH for 1 × 50 min.
8. Store larvae at -20 °C overnight before use.
   NOTE: *Larvae can be stored for six months at -20 °C.*

Buffer recipes for sample preparation

0.03% PTU stock solution (10×)
0.03% PTU

For 100 mL of solution
30 mg of 1-phenyl 2-thiourea powder
Fill up to 100 mL with egg H_2O
Heat solution at 50–60 °C overnight to dissolve powder

4% Paraformaldehyde (PFA)
4% PFA
1× PBS

For 25 mL of solution
1 g of PFA powder
25 mL of 1× PBS
Heat solution at 50–60 °C to dissolve powder

NOTE: *Avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the samples.*

Multiplexed HCR RNA-FISH v3.0 protocol

Detection stage

1. Transfer the required number of larvae for an experiment to a 2 mL eppendorf tube.
2. Rehydrate with a series of graded 1 mL MeOH/PBST washes for 5 min each at room temperature:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 5 × 100% PBST.
3. Treat 5 dpf larvae with 1 mL of proteinase K (30 μg/mL) for 45 min at room temperature.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage. Skip proteinase K treatment and postfixation (steps 3–6) for embryos 30 hpf and younger.*
4. Wash larvae two times with PBST (1 mL each) without incubation.
5. Postfix with 1 mL of 4 % PFA for 20 min at room temperature.
6. Wash larvae 5 × 5 min with 1 mL of PBST.
7. For each sample, move 8 larvae to a 1.5 mL eppendorf tube.
8. Pre-hybridize with 350 μL of 30% probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
9. Prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μL of 1 μM stock) to 500 μL of probe hybridization buffer at 37 °C.
   NOTE: *For single-molecule RNA imaging, use higher probe concentration (e.g., 16 nM) to increase probe hybridization yield. If desired, this approach can also be used to increase signal for subcellular quantitative RNA imaging.*
10. Remove the pre-hybridization solution and add the probe solution.
11. Incubate larvae overnight (12–16 h) at 37 °C.
12. Remove excess probes by washing embryos 4 × 15 min with 500 μL of 30% probe wash buffer at 37 °C.
    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 37 °C before use.*
13. Wash embryos 3 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify larvae with 350 μL of amplification buffer for 30 min at room temperature.
2. Separately prepare 30 pmol of hairpin H1 and 30 pmol of hairpin H2 by snap cooling 10 μL of 3 μM stock (heat at 95 °C for 90 seconds and cool to room temperature in a dark drawer for 30 min).
   NOTE: *HCR hairpins H1 and H2 are provided in hairpin storage buffer ready for snap cooling. H1 and H2 should be snap cooled in separate tubes.*
3. Prepare hairpin solution by adding all snap-cooled hairpins to 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate larvae overnight (12–16 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for a shorter period of time to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing with 500 μL of 5$\times$ SSCT at room temperature:
   (a) 2 $\times$ 5 min
   (b) 2 $\times$ 30 min
   (c) 1 $\times$ 5 min.

Sample mounting for microscopy

1. A chamber for mounting the larvae was made by aligning two stacks of Scotch tape (8 pieces per stack) 1 cm apart on a 25 mm $\times$ 75 mm glass slide.
2. Approximately 200 μL of 3% methyl cellulose mounting medium was added between the tape stacks on the slide.
3. Place larvae on the medium and orient for dorsal imaging.
4. Place a 22 mm $\times$ 22 mm No. 1 coverslip on top of the stacks to close the chamber.
5. A Zeiss 710 NLO inverted confocal microscope equipped with an LD C-Apochromat 40$\times$/1.1 W Korr M27 objective was used in our lab to acquire zebrafish images.

HCR reagents storage conditions

Probes, amplifiers, probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Amplification buffer should be stored at 4 °C. Keep these reagents on ice at all times during probe and amplifier preparation. Make sure all solutions are well mixed before use.

General buffer recipes for HCR RNA-FISH

PBST
1× PBS
0.1% Tween 20

For 50 mL of solution
5 mL of 10× PBS
500 μL of 10% Tween 20
Fill up to 50 mL with ultrapure H_2O

Proteinase K solution
30 μg/mL proteinase K

For 1 mL of solution
1.5 μL of 20 mg/mL proteinase K
Fill up to 1 mL with PBST

5× SSCT
5× sodium chloride sodium citrate (SSC)
0.1% Tween 20

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
Fill up to 40 mL with ultrapure H_2O

3% methyl cellulose
3% methyl cellulose

For 100 mL of solution
3 g of methyl cellulose powder
Fill up to 100 mL with ultrapure H_2O
Stir overnight at 4 °C with a stir bar to dissolve powder

Reagents and supplies

Paraformaldehyde (PFA) (Sigma Cat. # P6148)
1-phenyl-2-thiourea (PTU) (Sigma Cat. # P7629)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
10% Tween 20 solution (Bio-Rad Cat. # 161-0781)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
Methyl cellulose (Sigma Cat. # M0387)

Citation Notes

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- **10-plex HCR spectral imaging**
  HCR spectral imaging of any combination of 10 RNA and/or protein targets with 1-step quantitative HCR signal amplification for all targets simultaneously (Schulte *et al.*, 2024a).
  Software: HCR Imaging Python module including Dot Detection 2.0 and Unmix 1.0 packages.

- **HCR protein:protein complex imaging**
  Multiplexed, quantitative, high-resolution imaging of protein:protein complexes with/without HCR IF and/or HCR RNA-FISH with simultaneous HCR signal amplification for all protein:protein, protein, and RNA targets (Schulte *et al.*, 2024b).

- **HCR RNA-FISH/IF**
  A unified approach to multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free HCR signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf *et al.*, 2021).

- **HCR IF**
  Multiplexed, quantitative, high-resolution protein immunofluorescence (IF) in highly autofluorescent samples (e.g., FFPE brain tissue sections) (Schwarzkopf *et al.*, 2021).

- **HCR RNA-FISH (v3.0)**
  Automatic background suppression for dramatically enhanced performance (signal-to-background and quantitative precision) and ease-of-use (no probe set optimization for new targets) (Choi *et al.*, 2018). v3.0 supports three quantitative modes:

  qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context;

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput single-cell analysis;

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in an anatomical context.

  Protocols for v3.0 in diverse organisms are adapted from the zoo paper.
  Software: Dot Analysis 1.0 package.

- **Quantitative HCR (qHCR) imaging with subcellular resolution**
  Analog mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos. The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi *et al.*, 2018).
  Software: Read-out/Read-in 1.0 package.

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

bacteria in suspension	whole-mount zebrafish embryos and larvae
whole-mount fruit fly embryos	whole-mount chicken embryos
whole-mount worm larvae	whole-mount mouse embryos
whole-mount sea urchin embryos	FFPE human tissue sections

- **Digitial HCR (dHCR) imaging with single-molecule resolution**
  Digital mRNA absolute quantitation with single-molecule resolution in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **Multiplexed quantitative HCR (qHCR) northern blots**
  Simultaneous quantification of RNA target size and abundance with signal amplification for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**
  2nd generation HCR RNA-FISH technology (v2.0) using DNA probes and DNA HCR amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability (Choi *et al.*, 2014).

- **HCR RNA-FISH (v1.0)**
  1st generation HCR RNA-FISH technology (v1.0) using RNA probes and RNA HCR amplifiers: multiplexed mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi *et al.*, 2010).

- **HCR mechanism**
  The hybridization chain reaction (HCR) mechanism enables isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

We are happy to provide advice and technical support: support@moleculartechnologies.org.

– The Molecular Technologies Team

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