HCR RNA-FISH v3.0 protocol for whole-mount chicken embryos (*Gallus gallus domesticus*)

This protocol has been optimized for embryos at stage HH 8–11, for other stages additional optimization may be required.
Technical support: support@moleculartechnologies.org

Preparation of fixed whole-mount chicken embryos

1. Collect chick embryos on 3M paper circles and place in a petri dish containing Ringer's solution.
2. Transfer embryos into a new petri dish with fresh Ringer's solution.
   NOTE: *This is to rinse away egg yolk before fixation.*
3. Transfer into a petri dish containing 4% paraformaldehyde (PFA).
   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.*
4. Fix the samples at room temperature for 1 h.
5. Transfer embryos into a petri dish containing PBST.
6. Dissect the embryos off the filter paper. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL eppendorf tube containing PBST.
8. Nutate for 5 mins with the tube positioned horizontally in a small ice bucket.
9. Wash embryos two additional times with 2 mL of PBST, each time nutating for 5 min on ice.
10. Dehydrate embryos with 2 × 5 min washes of 2 mL methanol (MeOH) on ice.
11. Store embryos at -20 °C overnight before use.
    NOTE: *Embryos can be stored for six months at -20 °C.*
12. Transfer the required number of embryos for an experiment to a 2 mL eppendorf tube.
    NOTE: *Do not place more than 4 embryos in each 2 mL eppendorf tube.*
13. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 min each on ice:
    (a) 75% MeOH / 25% PBST
    (b) 50% MeOH / 50% PBST
    (c) 25% MeOH / 75% PBST
    (d) 100% PBST
    (e) 100% PBST.
14. Treat embryos with 2 mL of 10 μg/mL proteinase K solution for 2 min (stage HH 8) or 2.5 min (stages HH 10–11) 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.*
15. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
    CAUTION: *Use PFA with extreme care as it is a hazardous material.*
16. Wash embryos 2 × 5 min with 2 mL of PBST on ice.

17. Wash embryos with 2 mL of 50% PBST / 50% 5× SSCT for 5 min on ice.
18. Wash embryos with 2 mL of 5× SSCT for 5 min on ice.

Buffer recipes for sample preparation

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

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
10 μg/mL proteinase K

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

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

Multiplexed HCR RNA-FISH v3.0 protocol

Detection stage

1. For each sample, transfer 1-4 embryos to a 2 mL eppendorf tube.
   NOTE: *Do not place more than 4 embryos in each 2 mL eppendorf tube.*

2. Incubate embryos in 500 μL of 30% probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*

3. Prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μL of 1 μM stock) to 500 μL of 30% 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.*

4. Remove the pre-hybridization solution and add the probe solution.

5. Incubate embryos overnight (12–16 h) at 37 °C.

6. Remove excess probes by washing with 4 × 15 min with 1 mL of 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.*

7. Wash samples 2 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 500 μL of amplification buffer for 5 min at room temperature.
   NOTE: *equilibrate amplification buffer to room temperature before use.*

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 the embryos overnight (12–16 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for 90-min to ensure single-molecule dots are diffraction-limited.*

6. Remove excess hairpins by washing with 1 mL of 5× SSCT at room temperature:

   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

Sample mounting for microscopy

1. A chamber for mounting each embryo was made by aligning two stacks of double-sided tape (2 pieces per stack) 1 cm apart on a 25 mm $\times$ 75 mm glass slide.
2. Place an embryo between the tape stacks on the slide and remove as much solution as possible.
3. Align the embryo for dorsal imaging and carefully touch the slide with a kimwipe to further dry the area around the embryo.
4. Add two drops of SlowFade Gold antifade mountant on top of the embryo.
5. Place a 22 mm $\times$ 30 mm No. 1 coverslip on top of the stacks to close the chamber.
6. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25$\times$/0.8 Imm Corr DIC objective was used in our lab to acquire chicken 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

5× SSCT	For 40 mL of solution
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

Paraformaldehyde (PFA) (Sigma Cat. # P6148)
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)
50% Tween 20 (Life Technologies Cat. # 00-3005)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36937)

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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