In situ HCR v2.0 protocol for whole-mount fruit fly embryos (*Drosophila melanogaster*)

This protocol has been optimized for embryos at stage 4–6.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Collect Drosophila embryos and incubate with yeast paste (food source) until they reach stage 4-6 (approximately 3 h).
2. Rinse embryos into a mesh basket and wash excess yeast paste using DI H_2O.
3. Immerse the mesh basket into 100% bleach and wash for 2 min to dechorinate embryos.
4. Rinse the basket with DI H_2O.
5. Transfer embryos to a scintillation vial containing 8 mL of 4.5% formaldehyde fixation solution.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
6. Gently rock embryos in scintillation vial for 25 min on an orbital rocker.
   NOTE: *Rocking does not have to be very vigorous. However, embryos must move continuously within the heptane-fixative interphase to be exposed uniformly to the solvent and fixative.*
7. Remove the bottom liquid phase in the vial.
8. Add 8 mL of methanol (MeOH) and shake the vial hard for 1 min. Devitellinized embryos will sink to the bottom of the vial.
9. Remove all liquid and rinse 2 times in MeOH to remove debris.
10. Store embryos in 1 mL of MeOH at -20 °C before use.

Buffer recipes for sample preparation

4.5% formaldehyde fixation solution	For 8 mL of solution
4.5% formaldehyde	975 μL of 37% formaldehyde
0.5× PBS	400 μL of 10× PBS
25 mM EGTA	76 mg of EGTA
50% heptane	4 mL of heptane
	Fill up to 8 mL with ultrapure H_2O

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

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. For each sample, transfer 50 μL of embryos (using a cut pipet tip) to a 1.5 mL eppendorf tube.
2. Rinse embryos 4 times by quickly adding 1 mL of ethanol (EtOH), inverting the tube, and aspirating the supernatant.
3. Add 500 μL of EtOH and 250 μL of xylene and invert the tube.
   CAUTION: *Use xylene with care as it is a hazardous material.*
4. Add an additional 250 μL of xylene and invert the tube.
5. Add another 250 μL of xylene again and invert the tube. The tube should now contain 500 μL of EtOH and 750 μL of xylene.
6. Rock the tube at room temperature for 45–60 min. Longer rocking time is acceptable.
7. Aspirate the supernatant.
8. Rinse embryos once and wash 3 × 5 min with EtOH.
   NOTE: *All washes have a volume of 1 mL unless specified. All washes before pre-hybridization (step 16) are done with rocking.*
9. Rinse embryos once and wash 2 × 5 min with MeOH.
10. Wash with 50% MeOH / 50% PBST for 5 min.
11. Wash 1 × 10 min and 2 × 5 min with PBST.
12. Rock embryos in 1 mL of 4 μg/mL proteinase K solution at room temperature for 7 min.
    NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
13. Rinse embryos 2 times and wash 2 × 5 min with PBST.
14. Rock embryos in 4% formaldehyde post-fixation solution at room temperature for 25 min.
    CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
15. Rinse embryos and wash 5 × 5 min with PBST.
16. Pre-hybridize with 100 μL of probe hybridization buffer for 2 h at 65 °C.
    CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
17. Prepare probe solution by adding 0.2 pmol of each probe (0.2 μL of 1 μM stock per probe) to 100 μL of probe hybridization buffer at 45 °C.
18. Remove the pre-hybridization solution and add the probe solution.
19. Incubate embryos overnight (12–16 h) at 45 °C.
20. Remove excess probes by washing with probe wash buffer at 45 °C:
    (a) 2 × 5 min
    (b) 2 × 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 10 min at room temperature.
2. Separately prepare 6 pmol of hairpin H1 and 6 pmol of hairpin H2 by snap cooling 2 μL of 3 μM stock in hairpin storage buffer (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 100 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.
6. Remove excess hairpins by washing with 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

Sample mounting for microscopy

1. Place 25 μL of embryos on a 25 mm × 75 mm glass slide.
2. Add 1–2 drops of ProLong Gold antifade mountant onto the embryos and stir to mix using a pipette tip.
3. Place a 22 mm × 22 mm No. 1 coverslip on top and seal using clear nail polish.
4. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used in our lab to acquire fly images.

Buffer recipes for in situ HCR v2.0

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.

Proteinase K solution
4 μg/mL proteinase K

For 2 mL of solution
0.4 μL of 20 mg/mL proteinase K
Fill up to 2 mL with PBST

4% formaldehyde post-fixation solution
4% formaldehyde

For 2 mL of solution
216 μL of 37% formaldehyde
Fill up to 2 mL with PBST

Probe hybridization buffer
50% formamide
5× sodium chloride sodium citrate (SSC)
9 mM citric acid (pH 6.0)
0.1% Tween 20
50 μg/mL heparin
1× Denhardt's solution
10% dextran sulfate

For 40 mL of solution
20 mL formamide
10 mL of 20× SSC
360 μL 1 M citric acid, pH 6.0
400 μL of 10% Tween 20
200 μL of 10 mg/mL heparin
800 μL of 50× Denhardt's solution
8 mL of 50% dextran sulfate
Fill up to 40 mL with ultrapure H_2O

Probe wash buffer
50% formamide
5× sodium chloride sodium citrate (SSC)
9 mM citric acid (pH 6.0)
0.1% Tween 20
50 μg/mL heparin

For 40 mL of solution
20 mL formamide
10 mL of 20× SSC
360 μL 1 M citric acid, pH 6.0
400 μL of 10% Tween 20
200 μL of 10 mg/mL heparin
Fill up to 40 mL with ultrapure H_2O

Amplification buffer
5× sodium chloride sodium citrate (SSC)
0.1% Tween 20
10% dextran sulfate

For 40 mL of solution
10 mL of 20× SSC
400 μL of 10% Tween 20
8 mL of 50% dextran sulfate
Fill up to 40 mL with ultrapure H_2O

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

50% dextran sulfate
50% dextran sulfate

For 40 mL of solution
20 g of dextran sulfate powder
Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

Baker's yeast (VWR Cat. # IC10140001)
37% formaldehyde (Fisher Scientific Cat. # F79-4)
10× PBS (Life Technologies Cat.# AM9625)
Ethylene glycol tetra acetic acid (EGTA) (Sigma Cat. # E4378)
Heptane, HPLC-grade (EMD Millipore Cat. # HX0080-6)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Ethanol, 200 proof (VWR Cat. # V1001G)
Xylene (Mallinckrodt Chemicals Cat. # 8668-02)
20 mg/mL proteinase K solution (Life Technologies Cat. # 25530-049)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. > 500,000 (Sigma Cat. # D6001)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 22 mm No. 1 coverslip (VWR Cat. # 48366-067)
ProLong Gold antifade mountant (Life Technologies Cat. # P36930)

Citation Notes

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- **In situ HCR 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; qHCR flow cytometry – analog mRNA relative quantitation for high-throughput single-cell analysis; dHCR imaging – digital mRNA absolute quantitation. Protocols for v3.0 in diverse organisms are adapted from the zoo paper.

- **Quantitative HCR (qHCR) imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framework (Trivedi *et al.*, 2018).

- **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

- **Single-molecule HCR imaging**
  Single-molecule mRNA imaging 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).

- **In situ HCR v2.0**
  2nd generation in situ HCR 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).

- **Shielded covalent (SC) probes**
  Highly selective covalent capture of DNA and RNA targets, overcoming the longstanding tradeoff between selectivity and durable target capture (Vieregg *et al.*, 2013).

- **In situ HCR v1.0**
  1st generation in situ HCR 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).

- **Hybridization chain reaction (HCR) mechanism**
  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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