HCR™ RNA-FISH (v3.0) protocol for whole-mount fruit fly embryos

This protocol has not been optimized for all stages and should only be used as a template.

Technical Support
support@molecularinstruments.com

Safety Data Sheets (SDS)
www.molecularinstruments.com/safety-v3

Patents
www.molecularinstruments.com/patents

Ordering for Multiplex Experiment
Order one HCR™ RNA-FISH (v3.0) kit per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH (v3.0) kit for target mRNA1
  - HCR™ Probe (v3.0): target mRNA1 for use with amplifier B1
  - HCR™ Amplifier (v3.0): B1-647
  - HCR™ RNA-FISH Buffers (v3.0): HCR™ Probe Hybridization Buffer (v3.0), HCR™ Probe Wash Buffer (v3.0), HCR™ Amplifier Buffer (v3.0) (for use with all kits)
- HCR™ RNA-FISH (v3.0) kit for target mRNA2
  - HCR™ Probe (v3.0): target mRNA2 for use with amplifier B2
  - HCR™ Amplifier (v3.0): B2-488

Storage conditions

- Store HCR™ Probes (v3.0), HCR™ Amplifiers (v3.0), HCR™ Probe Hybridization Buffer (v3.0), and HCR™ Probe Wash Buffer (v3.0) at -20 °C.
- Store HCR™ Amplifier Buffer (v3.0) at 4 °C.
- On the bench top, keep stock solutions on ice.
- Make sure all solutions are well mixed before use.

Preparation of fixed whole-mount fruit fly embryos

1. Collect fly 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 1 mL of MeOH to remove debris.
   NOTE: *Embryos can be stored in 1 mL of MeOH at -20 °C before use.*
10. For each sample, transfer 50 μL of embryos (using a cut pipet tip) to a 1.5 mL tube.
11. Rinse embryos 4 times by quickly adding 1 mL of ethanol (EtOH), inverting the tube, and aspirating the supernatant.
12. 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.*
13. Add an additional 250 μL of xylene and invert the tube.
14. Add another 250 μL of xylene again and invert the tube.
    NOTE: *The tube should now contain 500 μL of EtOH and 750 μL of xylene.*
15. Rock the tube at room temperature for 45–60 min. Longer rocking time is acceptable.
16. Aspirate the supernatant.
17. Rinse embryos once and wash 3 $\times$ 5 min with EtOH.
    NOTE: *All washes have a volume of 1 mL unless specified. All washes before pre-hybridization (step 1) are performed with rocking.*
18. Rinse embryos once and wash 2 $\times$ 5 min with MeOH.

19. Wash with 50% MeOH / 50% PBST for 5 min.
20. Wash 1 × 10 min and 2 × 5 min with PBST.
21. 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.*
22. Rinse embryos 2 times and wash 2 × 5 min with PBST.
23. Rock embryos in 4% formaldehyde post-fixation solution at room temperature for 25 min.
24. Rinse embryos and wash 5 × 5 min with PBST.

Multiplexed HCR™ RNA-FISH (v3.0) protocol

Detection stage

1. Pre-hybridize embryos in 200 μL of HCR™ Probe Hybridization Buffer (v3.0) for 30 min at 37 °C.
   CAUTION: *HCR™ Probe Hybridization Buffer (v3.0) contains formamide, a hazardous material.*

2. Prepare probe solution by adding 0.8 pmol of each HCR™ Probe (v3.0) (e.g. 0.8 μL of 1 μM stock) to 200 μL of HCR™ Probe Hybridization Buffer (v3.0) 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 qHCR imaging.*

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

4. Incubate embryos overnight (>12 h) at 37 °C.

5. Remove excess probes by washing embryos 4 × 15 min with 1 mL of HCR™ Probe Wash Buffer (v3.0) at 37 °C.
   CAUTION: *HCR™ Probe Wash Buffer (v3.0) contains formamide, a hazardous material.*
   NOTE: *pre-heat HCR™ Probe Wash Buffer (v3.0) to 37 °C before use.*

6. Wash samples 2 × 5 min with 1 mL of 5× SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 1 mL of HCR™ Amplifier Buffer (v3.0) for 10 min at room temperature.
   NOTE: *equilibrate HCR™ Amplifier Buffer (v3.0) to room temperature before use.*

2. Separately prepare 6 pmol of hairpin h1 and 6 pmol of hairpin h2 by snap cooling 2 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: *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 snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL of HCR™ Amplifier Buffer (v3.0) at room temperature.

4. Remove the pre-amplification solution and add the hairpin solution.

5. Incubate the embryos overnight (>12 h) in the dark at room temperature.
   NOTE: *For single-molecule RNA imaging, amplify for a shorter period of time 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

7. Samples can be stored at 4 °C protected from light before microscopy.

Buffer recipes

4.5% formaldehyde fixation solution
4.5% formaldehyde
0.5× PBS
25 mM EGTA
50% heptane

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

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

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

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

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

HCR™ Technology Citation Notes

For citation, please select from the list below as appropriate for your application:

- **10-Plex HCR™ Spectral Imaging**
  HCR™ RNA-FISH/IF enables quantitative high-resolution imaging of 10 RNA and/or protein targets with 1-step HCR™ signal amplification for all targets simultaneously. The method is suitable even for whole-mounts and delicate samples as it requires no repeated staining, imaging, registration, or stripping (Schulte et al., 2024).

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

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

- **HCR™ RNA-FISH**
  - Third-generation HCR™ RNA-FISH (v3.0) enables multiplex, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol for dramatically enhanced performance (signal-to-background, subcellular quantitative RNA imaging precision, single-molecule quantitative RNA imaging fidelity) and ease-of-use (no probe set optimization for new targets and organisms) (Choi et al., 2018).
  - Second-generation HCR™ RNA-FISH (v2.0) using DNA HCR™ Probes and DNA HCR™ Amplifiers: 10× increase in signal, 10× reduction in cost, dramatic increase in reagent durability (Choi et al., 2014).
  - First-generation HCR™ RNA-FISH (v1.0) using RNA HCR™ Probes and RNA HCR™ Amplifiers: multiplex mRNA imaging in whole-mount vertebrate embryos with simultaneous signal amplification for up to 5 target mRNAs (Choi et al., 2010).

- **Subcellular Quantitative RNA and Protein Imaging**
  HCR™ RNA-FISH enables analog relative quantitation of RNA and/or protein targets with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos) (Trivedi et al., 2018, Choi et al., 2018, Schwarzkopf et al., 2021).

- **Single-Molecule Quantitative RNA Imaging**
  HCR™ RNA-FISH enables digital RNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah et al., 2016, Choi et al., 2018).

- **Read-Out/Read-In Analysis Framework**
  The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi et al., 2018).

- **Protocols in Diverse Sample Types**
  Protocols for HCR™ RNA-FISH and/or IF in diverse sample types are adapted from the zoo paper (Choi et al., 2016):
  - bacteria in suspension

- FFPE human tissue sections
- generic sample in solution
- generic sample on a slide
- mammalian cells on a slide
- mammalian cells in suspension
- whole-mount chicken embryos
- whole-mount fruit fly embryos
- whole-mount mouse embryos
- whole-mount nematode larvae
- whole-mount sea urchin embryos
- whole-mount zebrafish embryos and larvae

- **HCR™ RNA Flow Cytometry**
  HCR™ RNA Flow Cytometry enables analog RNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria without the need to engineer reporter lines (Choi et al., 2018).

- **HCR™ Northern Blots**
  HCR™ Northern Blots enable simultaneous quantification of RNA target size and abundance with automatic background suppression throughout the protocol (Schwarzkopf & Pierce, 2016).

- **HCR™ Amplifiers**
  HCR™ Amplifiers enable multiplex, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings (Dirks & Pierce, 2004).