

HCR RNA-FISH protocol for whole-mount fruit fly embryos (*Drosophila melanogaster*)

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

Technical support

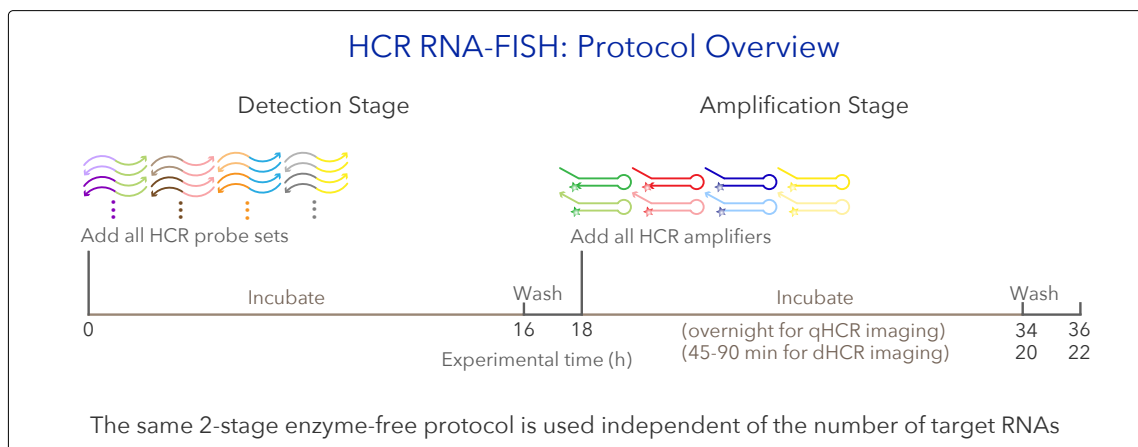
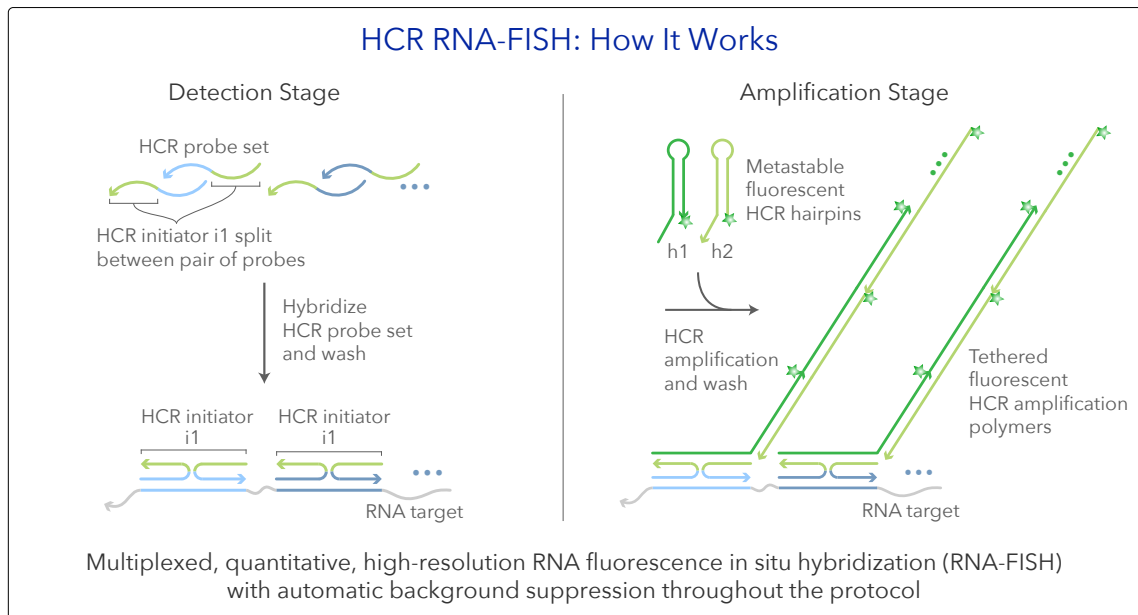
support@molecularinstruments.com

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HCR RNA-FISH

Multiplexed, quantitative, high-resolution RNA imaging

Multiplexed Experiment

- Order one HCR RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR bundle for target mRNA1
 - HCR split-initiator probe set: target mRNA1 for use with amplifier B1
 - HCR amplifier: B1-647
 - HCR RNA-FISH buffers: probe hybridization buffer, probe wash buffer, amplification buffer (for use with all bundles)
- HCR bundle for target mRNA2
 - HCR split-initiator probe set: target mRNA2 for use with amplifier B2
 - HCR amplifier: B2-488

Storage conditions

- Store HCR probe sets, HCR amplifiers, HCR probe hybridization buffer, and HCR probe wash buffer at -20 °C.
- Store HCR amplification buffer 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 H₂O.
3. Immerse the mesh basket into 100% bleach and wash for 2 min to dechorinate embryos.
4. Rinse the basket with DI H₂O.
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 × 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 × 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 protocol

Detection stage

1. Pre-hybridize embryos in 200 μL of probe hybridization buffer for 30 min at 37 °C.
CAUTION: probe hybridization buffer contains formamide, a hazardous material.
2. Prepare probe solution by adding 0.8 pmol of each probe set (e.g. 0.8 μL of 1 μM stock) to 200 μL of probe hybridization buffer at 37 °C.
NOTE: For dHCR 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 \times 15 min with 1 mL of probe wash buffer at 37 °C.
CAUTION: probe wash buffer contains formamide, a hazardous material.
NOTE: pre-heat probe wash buffer to 37 °C before use.
6. Wash samples 2 \times 5 min with 1 mL of 5 \times SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 10 min at room temperature.
NOTE: equilibrate amplification buffer 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: 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 snap-cooled h1 hairpins and snap-cooled h2 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 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 1 mL of 5 \times SSCT at room temperature:
 - (a) 2 \times 5 min
 - (b) 2 \times 30 min
 - (c) 1 \times 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 μ L of 37% formaldehyde
400 μ L of 10× PBS
76 mg of EGTA
4 mL of heptane
Fill up to 8 mL with ultrapure H₂O

Proteinase K solution

4 μ g/mL proteinase K

For 2 mL of solution

0.4 μ L 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 μ L 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 μ L of 10% Tween 20
Fill up to 50 mL with ultrapure H₂O

5× SSCT

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

For 40 mL of solution

10 mL of 20× SSC
400 μ L of 10% Tween 20
Fill up to 40 mL with ultrapure H₂O

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:

- **HCR IHC + HCR RNA-FISH**

[HCR IHC + HCR RNA-FISH](#) enables a unified approach to multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) and RNA fluorescence in situ hybridization (RNA-FISH), with quantitative 1-step enzyme-free HCR signal amplification performed for all protein and RNA targets simultaneously ([Schwarzkopf et al., 2021](#)).

- **HCR IHC**

[HCR IHC](#) enables multiplexed, quantitative, high-resolution protein immunohistochemistry (IHC) in highly autofluorescent samples (e.g., FFPE brain tissue sections) ([Schwarzkopf et al., 2021](#)).

- **HCR RNA-FISH (v3.0)**

Third-generation [HCR RNA-FISH \(v3.0\)](#) enables multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with [automatic background suppression throughout the protocol](#) for dramatically enhanced performance (signal-to-background, qHCR precision, dHCR fidelity) and ease-of-use (no probe set optimization for new targets and organisms) ([Choi et al., 2018](#)). Quantitative analysis modes:

- [qHCR RNA imaging](#): analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
- [dHCR RNA imaging](#): digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
- [qHCR RNA flow cytometry](#): analog mRNA relative quantitation for high-throughput expression profiling of mammalian cells and bacteria.

[Protocols for HCR RNA-FISH \(v3.0\)](#) in diverse organisms are adapted from the Zoo paper.

- **qHCR RNA imaging**

[qHCR RNA imaging](#) enables mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples (e.g., whole-mount vertebrate embryos). The [read-out/read-in analysis framework](#) enables bidirectional quantitative discovery in an anatomical context ([Trivedi et al., 2018](#)).

- **Zoo paper**

Protocols for multiplexed mRNA imaging in diverse sample types ([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

- **dHCR imaging**

[dHCR RNA imaging](#) enables 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](#)).

- **qHCR northern blots**

[qHCR northern blots](#) enable multiplexed quantification of RNA target size and abundance for up to 5 target RNAs ([Schwarzkopf & Pierce, 2016](#)).

- **HCR RNA-FISH (v2.0)**

Second-generation in situ HCR RNA-FISH technology (v2.0) using DNA HCR probes and DNA HCR 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)**

First-generation HCR RNA-FISH technology (v1.0) using RNA HCR 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 multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings ([Dirks & Pierce, 2004](#)).

HCR Technology References

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