

## 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

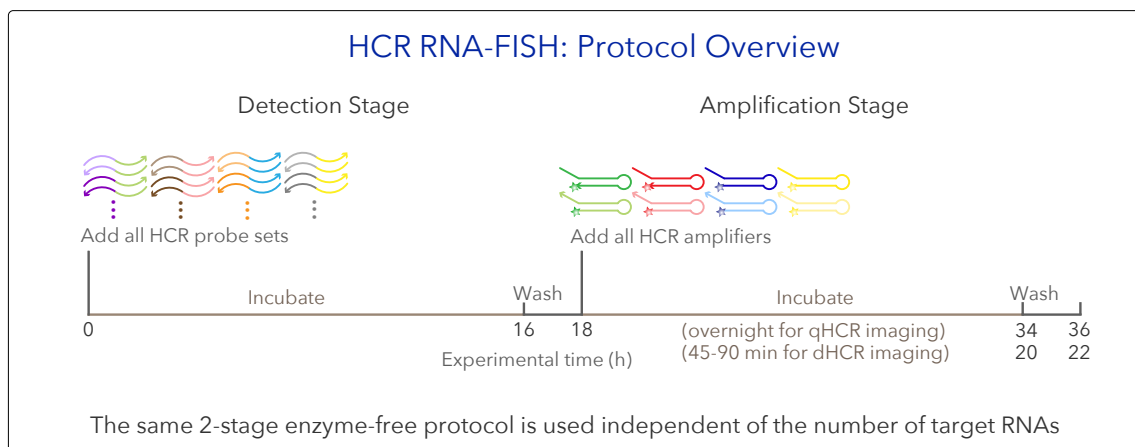
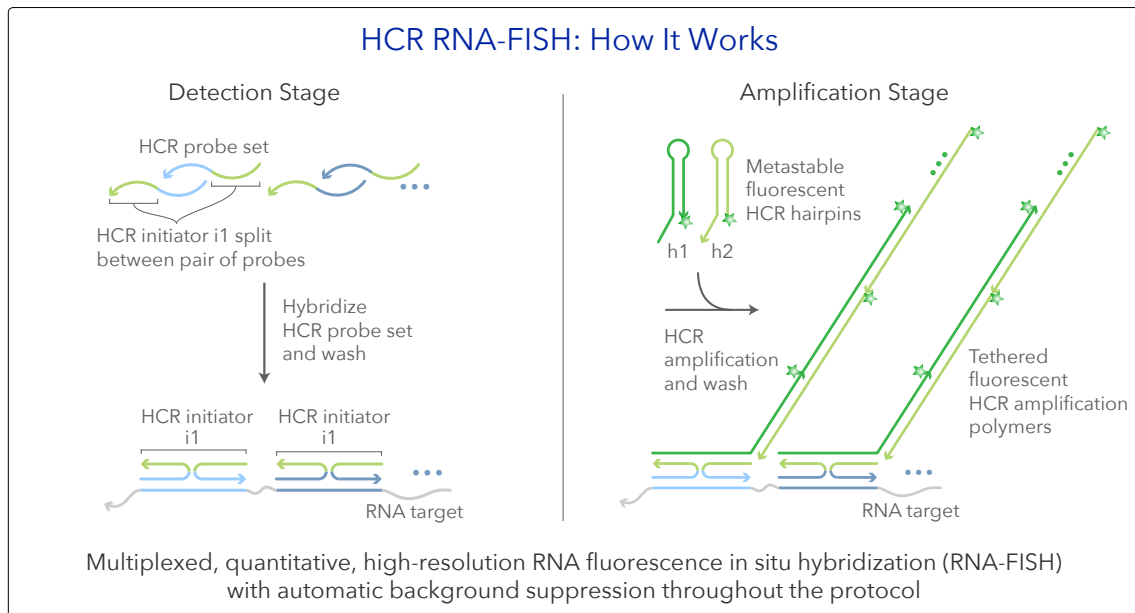
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## **HCR RNA-FISH**

*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 H<sub>2</sub>O.
3. Immerse the mesh basket into 100% bleach and wash for 2 min to dechorionate embryos.
4. Rinse the basket with DI H<sub>2</sub>O.
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  $\mu\text{L}$  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  $\mu\text{L}$  of 1  $\mu\text{M}$  stock) to 200  $\mu\text{L}$  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 (12–16 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  $\mu\text{L}$  of 3  $\mu\text{M}$  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  $\mu\text{L}$  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 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  $\mu$ L of 37% formaldehyde  
400  $\mu$ L of 10× PBS  
76 mg of EGTA  
4 mL of heptane  
Fill up to 8 mL with ultrapure H<sub>2</sub>O

### Proteinase K solution

4  $\mu$ g/mL proteinase K

### For 2 mL of solution

0.4  $\mu$ 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  $\mu$ 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  $\mu$ L of 10% Tween 20  
Fill up to 50 mL with ultrapure H<sub>2</sub>O

### 5× SSCT

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

### For 40 mL of solution

10 mL of 20× SSC  
400  $\mu$ L of 10% Tween 20  
Fill up to 40 mL with ultrapure H<sub>2</sub>O

*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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