

HCR™ RNA-FISH protocol for whole-mount zebrafish embryos and larvae (*Danio rerio*)

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

Technical support

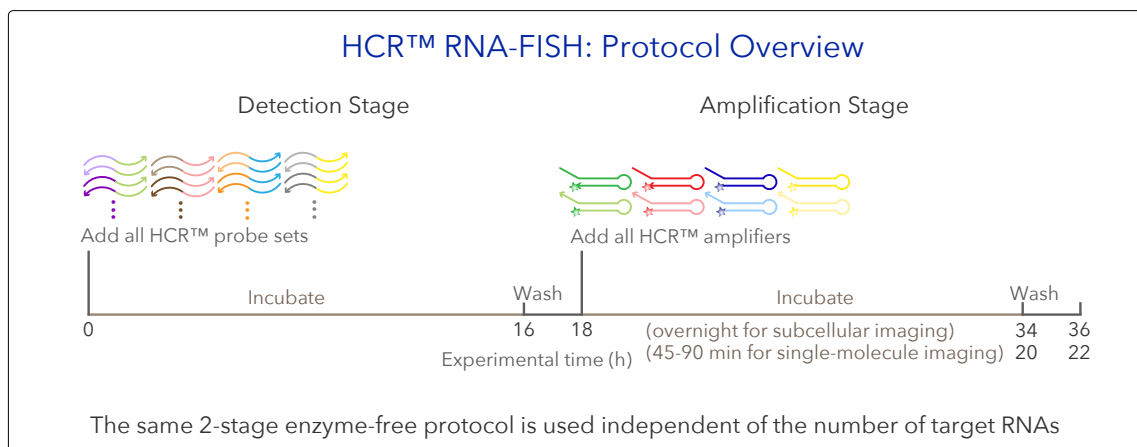
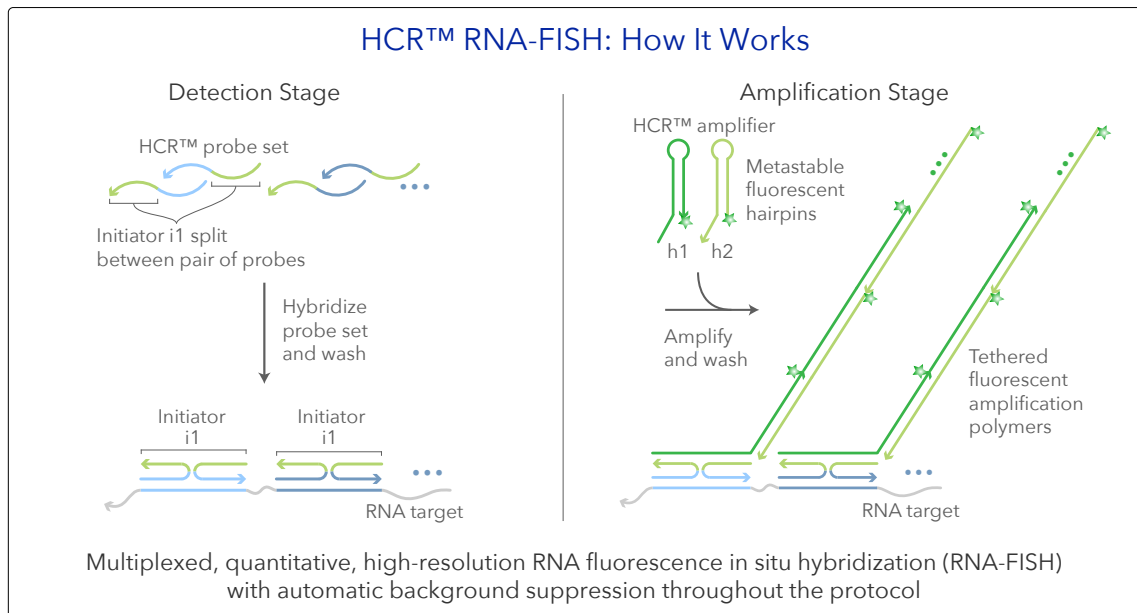
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HCR™ RNA-FISH

Multiplexed, quantitative, high-resolution RNA imaging

Multiplexed Experiment

- Order one HCR™ RNA-FISH bundle per target RNA

Example 2-Plex Experiment

- HCR™ RNA-FISH bundle for target mRNA1
 - HCR™ 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™ RNA-FISH bundle for target mRNA2
 - HCR™ probe set: target mRNA2 for use with amplifier B2
 - HCR™ amplifier: B2-488

Storage conditions

- Store HCR™ probe sets, HCR™ amplifiers, HCR™ RNA-FISH probe hybridization buffer, and HCR™ RNA-FISH 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 zebrafish embryos and larvae

1. Collect zebrafish embryos and incubate at 28 °C in a petri dish with egg H₂O.
2. Exchange egg H₂O with egg H₂O containing 0.003% of 1-phenyl 2-thiourea (PTU) when embryos reach 12 hpf.
NOTE: PTU treatment is not necessary if working with embryos younger than 30 hpf.
NOTE: PTU inhibits melanogenesis but can be toxic at high concentrations. PTU treatment must start before the initial pigmentation occurs as PTU does not remove pigment that has already formed. PTU treatment is not necessary for nacre embryos used in this paper.
3. Replace with fresh egg H₂O containing 0.003% of PTU everyday until the larvae reach 5 dpf (days post-fertilization).
4. Transfer ~40 embryos/larvae (5 dpf) to a 2 mL eppendorf tube and remove excess egg H₂O.
5. Fix embryos/larvae in 2 mL of 4% paraformaldehyde (PFA) for 24 h at 4 °C.
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.
6. Wash embryos/larvae 3 × 5 min with 1 mL of 1× phosphate-buffered saline (PBS) to stop the fixation.
NOTE: avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the embryos/larvae.
7. Dehydrate and permeabilize with a series of methanol (MeOH) washes (1 mL each):
 - (a) 100% MeOH for 4 × 10 min
 - (b) 100% MeOH for 1 × 50 min.
8. Store embryos/larvae at -20 °C overnight before use.
NOTE: Embryos/larvae can be stored for six months at -20 °C.
9. Transfer the required number of embryos/larvae for an experiment to a 2 mL eppendorf tube.
10. Rehydrate with a series of graded 1 mL MeOH/PBST washes for 5 min each at room temperature:
 - (a) 75% MeOH / 25% PBST
 - (b) 50% MeOH / 50% PBST
 - (c) 25% MeOH / 75% PBST
 - (d) 5 × 100% PBST.
11. Treat 5 dpf embryos/larvae with 1 mL of proteinase K (30 µg/mL) for 45 min 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. Skip proteinase K treatment and postfixation (steps 11–14) for embryos 30 hpf and younger.

12. Wash embryos/larvae two times with PBST (1 mL each) without incubation.
13. Postfix with 1 mL of 4% PFA for 20 min at room temperature.
14. Wash embryos/larvae 5 × 5 min with 1 mL of PBST.

Multiplexed HCR™ RNA-FISH protocol

Detection stage

1. For each sample, transfer 8 embryos/larvae to a 1.5 mL tube.
2. Pre-hybridize with 500 μL of 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 set (e.g. 2 μL of 1 μM stock) to 500 μL of 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/larvae overnight (>12 h) at 37 °C.
6. Remove excess probes by washing embryos/larvae 4 \times 15 min with 500 μL 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.
7. Wash embryos/larvae 2 \times 5 min with 5 \times SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos/larvae with 500 μL of amplification buffer for 30 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: 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 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos/larvae 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 500 μL 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

6% PTU stock solution

6% PTU

For 100 mL of solution

6 g of 1-phenyl 2-thiourea powder

Fill up to 100 mL with egg H₂O

Heat solution at 50–60 °C overnight to dissolve powder

0.3% PTU in egg H₂O

0.3% PTU

For 50 mL of solution

2.5 mL of 6% PTU

Fill up to 50 mL with with egg H₂O

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 H₂O

Proteinase K solution

30 μg/mL proteinase K

For 1 mL of solution

1.5 μL of 20 mg/mL proteinase K

Fill up to 1 mL with PBST

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 H₂O

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

HCR™ Technology Citation Notes

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

- **HCR™ IF + HCR™ RNA-FISH**

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

- **HCR™ IF**

[HCR™ IF](#) enables 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)**

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, 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](#)). Quantitative analysis modes:

- [Subcellular quantitative RNA imaging](#): analog mRNA relative quantitation with subcellular resolution in the anatomical context of thick autofluorescent samples.
- [Single-molecule quantitative RNA imaging](#): digital mRNA absolute quantitation with single-molecule resolution in the anatomical context of thick autofluorescent samples.
- [Quantitative 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.

- **Subcellular quantitative RNA imaging**

[Subcellular quantitative 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

- **Single-molecule quantitative RNA imaging**

[Single-molecule quantitative 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](#)).

- **HCR™ northern blots**

[HCR™ 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™ 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™ technology**

[HCR™ amplifiers](#) enable multiplexed, quantitative, 1-step, isothermal, enzyme-free signal amplification in diverse technological settings ([Dirks & Pierce, 2004](#)).