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

**Safety data sheets (SDS)**
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HCR RNA-FISH: How It Works

**Detection Stage**
- HCR probe set
- HCR initiator i1 split between pair of probes
- Hybridize HCR probe set and wash
- HCR initiator i1
- HCR initiator i1
- RNA target

**Amplification Stage**
- Metastable fluorescent HCR hairpins
- HCR amplification and wash
- Tethered fluorescent HCR amplification polymers

Multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) with automatic background suppression throughout the protocol

HCR RNA-FISH: Protocol Overview

**Detection Stage**
- Add all HCR probe sets
- Incubate 0-16 h
- Wash 18 h

**Amplification Stage**
- Add all HCR amplifiers
- Incubate (overnight for qHCR imaging) 34 h
- Wash (45-90 min for dHCR imaging) 36 h

The same 2-stage enzyme-free protocol is used independent of the number of target RNAs
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.
   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.
   \textit{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.
   \textit{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 × 15 min with 1 mL of probe wash buffer at 37 °C.
   \textit{CAUTION: probe wash buffer contains formamide, a hazardous material.}
   \textit{Note: pre-heat probe wash buffer 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 amplification buffer for 10 min at room temperature.
   \textit{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).
   \textit{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 (12–16 h) in the dark at room temperature.
   \textit{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× SSCT at room temperature:
   \begin{itemize}
   \item[(a)] 2 × 5 min
   \item[(b)] 2 × 30 min
   \item[(c)] 1 × 5 min
   \end{itemize}

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

#### 4.5% formaldehyde fixation solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5% formaldehyde</td>
<td>975 µL of 37% formaldehyde</td>
</tr>
<tr>
<td>0.5× PBS</td>
<td>400 µL of 10× PBS</td>
</tr>
<tr>
<td>25 mM EGTA</td>
<td>76 mg of EGTA</td>
</tr>
<tr>
<td>50% heptane</td>
<td>4 mL of heptane</td>
</tr>
</tbody>
</table>

Fill up to 8 mL with ultrapure H₂O

#### Proteinase K solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 µg/mL proteinase K</td>
<td>0.4 µL of 20 mg/mL proteinase K</td>
</tr>
</tbody>
</table>

Fill up to 2 mL with PBST

#### 4% formaldehyde post-fixation solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4% formaldehyde</td>
<td>216 µL of 37% formaldehyde</td>
</tr>
</tbody>
</table>

Fill up to 2 mL with PBST

#### PBST

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1× PBS</td>
<td>5 mL of 10× PBS</td>
</tr>
<tr>
<td>0.1% Tween 20</td>
<td>500 µL of 10% Tween 20</td>
</tr>
</tbody>
</table>

Fill up to 50 mL with ultrapure H₂O

#### 5× SSCT

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5× sodium chloride</td>
<td>10 mL of 20× SSC</td>
</tr>
<tr>
<td>sodium citrate (SSC)</td>
<td>400 µL of 10% Tween 20</td>
</tr>
<tr>
<td>0.1% Tween 20</td>
<td>Fill up to 40 mL with ultrapure H₂O</td>
</tr>
</tbody>
</table>

**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


