HCR™ Gold RNA-FISH User Guide

This User Guide enables HCR™ Gold RNA-FISH for multiplex, quantitative, high-resolution RNA imaging in diverse organisms and sample types. Technical support: support@molecularinstruments.com

Contents

HCR™ Gold RNA-FISH Kit Information **2**
- Ordering for Multiplex Experiment . . . 2
- Example 2-Plex Experiment . . . 2
- Safety Data Sheets (SDS) . . . 2
- Patents . . . 2
- HCR™ Gold RNA-FISH Storage Conditions and Shelf Life . . . 3
- User-Supplied Materials . . . 3

HCR™ Gold RNA-FISH Workflow **4**
- Sample Preparation . . . 4
- Probe Hybridization . . . 4
- Amplification . . . 4
- Counterstain and Imaging . . . 4

HCR™ Gold RNA-FISH Protocol: Sample on a Slide **5**
- Sample Preparation . . . 5
- Probe Hybridization . . . 5
- Amplification . . . 5

HCR™ Gold RNA-FISH Protocol: Sample in Solution **7**
- Sample Preparation . . . 7
- Probe Hybridization . . . 7
- Amplification . . . 7

Sample Preparation Protocols **9**
- FFPE Tissue Sections on a Slide . . . 10
- Fresh/Fixed Frozen Tissue Sections on a Slide . . . 12
- Whole-Mount Zebrafish Embryos and Larvae in Solution . . . 13
- Whole-Mount Mouse Embryos in Solution . . . 15
- Whole-Mount Chicken Embryos in Solution . . . 17
- Mammalian Cells on a Chambered Slide . . . 19

Common Recipes **20**

Frequently Asked Questions (FAQ) **22**

HCR™ Technology Citation Notes **24**

HCR™ RNA-FISH User Examples by Organism and Sample Type **26**

Selected HCR™ RNA-FISH User Publications **30**

HCR™ Gold RNA-FISH Kit Information

Ordering for Multiplex Experiment

- Order one HCR™ Gold RNA-FISH kit per target RNA

Example 2-Plex Experiment

- HCR™ Gold RNA-FISH kit for target RNA1
  - HCR™ HiFi Probe: for target RNA1 for use with amplifier X3
  - HCR™ HiFi Probe Hybridization Buffer (for use with all kits)
  - HCR™ HiFi Probe Wash Buffer (for use with all kits)
  - HCR™ Gold Amplifier: X3 with label 647
  - HCR™ Gold Amplifier Buffer (for use with all kits)
  - HCR™ Gold Amplifier Wash Buffer (for use with all kits)
- HCR™ Gold RNA-FISH kit for target RNA2
  - HCR™ HiFi Probe: for target RNA2 for use with amplifier X1
  - HCR™ Gold Amplifier: X1 with label 488

Safety Data Sheets (SDS)

- www.molecularinstruments.com/safety

Patents

- www.molecularinstruments.com/patents

HCR™ Gold RNA-FISH Storage Conditions and Shelf Life

Upon receiving your HCR™ Gold RNA-FISH kit, please check storage conditions for each reagent. HCR™ reagents should be thawed and mixed before use. We recommend aliquoting the HCR™ HiFi Probes and HCR™ Gold Amplifiers to minimize freeze-thaw cycles and maximize shelf life. On the bench top, keep stock solutions on ice. Make sure all solutions are well mixed before use.

HCR™ Reagent	Storage Temperature (°C)	Storage Condition	Shelf Life (yr)	Comments
HCR™ HiFi Probe	−20	—	2	
HCR™ HiFi Probe Hybridization Buffer	−20	—	1	
HCR™ HiFi Probe Wash Buffer	4	—	1	Provided at 4×; dilute to 1× with UltraPure H_2O before use
HCR™ Gold Amplifier	−20	Shielded from light	2	Comes in two separate tubes (h1 and h2)
HCR™ Gold Amplifier Buffer	4	—	1	
HCR™ Gold Amplifier Wash Buffer	4	—	1	Provided at 4×; dilute to 1× with UltraPure H_2O before use

User-Supplied Materials

Reagent[†]	Supplier	Comments
UltraPure H_2O	Any	Type I water
ProLong™ Gold Antifade Mountant with DAPI	ThermoFisher	This is our recommended antifade mountant but any antifade mountant, with or without DAPI, is acceptable (e.g., Fluoromount-G).

[†]All user-supplied reagents should be DNase- and RNase-free.

HCR™ Gold RNA-FISH Workflow

Below is a general overview of the steps involved and their purposes in the HCR™ Gold RNA-FISH assay.

Sample Preparation

1. **Fixation:** Preserve morphology and cellular components of a sample (e.g., DNA, RNA, proteins). Fixation is sensitive to several factors including type of fixative, concentration, and duration, and must be optimized for each sample type.
2. **Permeabilization:** Ensure the sample is sufficiently permeable for HCR™ reagents to diffuse into and out of the sample.

Probe Hybridization

3. **Pre-Hybridization:** Pre-condition the sample in HCR™ HiFi Probe Hybridization Buffer to block non-specific binding sites and minimize potential background signal. The buffer used in this step is identical to the probe hybridization step, but it does not contain any HCR™ HiFi Probe. Minimizing background is critical for maximizing signal-to-background.
4. **Probe Hybridization:** Introduce HCR™ HiFi Probes complementary to the RNA targets of interest to localize initiation sites at cognate targets.
5. **Probe Washing:** Remove any unbound or non-specifically bound probes through a series of washing steps to ensure they do not generate amplified background in the next stage.

Amplification

6. **Pre-Amplification:** Equilibrate in HCR™ Gold Amplifier Buffer to facilitate diffusion of HCR™ Gold Amplifiers into the sample.
7. **Amplification:** Each HCR™ Gold Amplifier comprises two hairpins (h1 and h2) that are snap-cooled separately before use to ensure proper folding. Introduce HCR™ Gold Amplifiers that diffuse to the cognate target where the initiation sites trigger growth of tethered fluorescent amplification polymers.
8. **Amplifier Washing:** Remove any unbound HCR™ Gold Amplifiers from the sample through a series of washing steps to minimize background.

Counterstain and Imaging

9. **Counterstain (optional):** Apply a counterstain such as DAPI or Hoechst to visualize nuclei in the sample. Counterstaining provides morphological context to facilitate image analysis.
10. **Imaging:** Use an appropriate mounting solution to safeguard the integrity of the HCR™ Gold RNA-FISH signal. Mounting media also serves to secure the sample during imaging to prevent it from drying and helps match the refractive index of an objective lens. Image using a fluorescent microscope (e.g., epifluorescence, confocal, light sheet) suitable for your sample type.

HCR™ Gold RNA-FISH Protocol: Sample on a Slide

This protocol is suitable for use with FFPE tissue sections, fresh/frozen tissue sections, FFPE cell pellets, adherent cells, etc on a slide. This protocol has not been validated for all sample types and should only be used as a template.

Sample Preparation

Samples should be prepared in the same manner as for traditional in situ hybridization up to the probe hybridization step. Then proceed with the protocol described below.

Probe Hybridization

1. Pre-heat a humidified chamber and HCR™ HiFi Probe Hybridization Buffer to 37 °C.
   CAUTION: *HCR™ HiFi Probe Hybridization Buffer contains formamide, a hazardous material.*
2. Pre-hybridize by adding 200 µL of HCR™ HiFi Probe Hybridization Buffer on top of sample and incubating at 37 °C for 10 min inside the humidified chamber.
3. Prepare probe solution by adding 2 µL of each HCR™ HiFi Probe to 100 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
4. Remove the pre-hybridization solution and drain excess buffer on slide by blotting edges on a Kimwipe.
5. Hybridize by adding 50–100 µL of the probe solution on top of sample, placing a coverslip on sample, and incubating at 37 °C for >3 h in the humidified chamber.
   NOTE: *Use enough probe solution to fully cover sample.*
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for thick brain slices).*
6. Pre-heat HCR™ HiFi Probe Wash Buffer to 37 °C.
   NOTE: *HCR™ HiFi Probe Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
7. Immerse slide in HCR™ HiFi Probe Wash Buffer at 37 °C to float off coverslip.
8. Remove excess probe by immersing 4 × 15 min in HCR™ HiFi Probe Wash Buffer at 37 °C. Dry slide by blotting edges on a Kimwipe.

Amplification

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. Drain slide by blotting edges on a Kimwipe and wipe around the sample with another Kimwipe.
3. Pre-amplify by adding 200 µL of HCR™ Gold Amplifier Buffer on top of sample and incubating at room temperature for 30 min in a humidified chamber.
4. Separately prepare 2 µL of hairpin h1 and 2 µL of hairpin h2. Snap cool each hairpin separately by heating at 95 °C for 90 sec and cooling to room temperature in a dark drawer for 30 min.
5. Prepare amplifier solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 µL of HCR™ Gold Amplifier Buffer at room temperature.

6. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.

7. Add 50–100 µL of the amplifier solution on top of the sample.
   NOTE: *Use enough amplifier solution to fully cover sample.*

8. Place a coverslip on the sample and incubate at room temperature for >3 h in the humidified chamber in the dark.
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for thick brain slices).*
   NOTE: *For single-molecule imaging, decrease incubation time (e.g., 90 min) if you want single-molecule dots to be diffraction-limited in size.*

9. Pre-warm HCR™ Gold Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Gold Amplifier Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*

10. Immerse slide in HCR™ Gold Amplifier Wash Buffer at room temperature to float off coverslip.

11. Remove excess amplifier by immersing 4 × 15 min in HCR™ Gold Amplifier Wash Buffer at room temperature in the dark. Dry slide by blotting edges on a Kimwipe.

12. Add antifade mounting reagent on top of sample and place coverslip on sample.
    NOTE: *Use antifade mounting reagent with a counterstain (e.g., DAPI) if preferred.*

13. Store sample at 4 °C and shield from light prior to imaging.

HCR™ Gold RNA-FISH Protocol: Sample in Solution

This protocol is suitable for use with whole-mount embryos, larvae, thick brain slices, etc in a tube or for adherent mammalian cells on a chambered slide. This protocol has not been validated for all sample types and should only be used as a template.

Sample Preparation

Samples should be prepared in the same manner as for traditional in situ hybridization up to the probe hybridization step. Then proceed with the protocol described below.

Probe Hybridization

1. Pre-heat HCR™ HiFi Probe Hybridization Buffer to 37 °C.
   CAUTION: *HCR™ HiFi Probe Hybridization Buffer contains formamide, a hazardous material.*
2. Pre-hybridize samples in 500 µL of HCR™ HiFi Probe Hybridization Buffer for 30 min at 37 °C.
   NOTE: *Use enough HCR™ HiFi Probe Hybridization Buffer to immerse sample.*
3. Prepare probe solution by adding 10 µL of each HCR™ HiFi Probe to 500 µL of HCR™ HiFi Probe Hybridization Buffer at 37 °C.
4. Remove the pre-hybridization solution and add the probe solution.
5. Incubate sample for >3 h at 37 °C.
   NOTE: *Use enough probe solution to immerse sample.*
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for whole-mount vertebrate embryos).*
6. Pre-heat HCR™ HiFi Probe Wash Buffer to 37 °C.
   NOTE: *HCR™ HiFi Probe Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*
7. Remove excess probe by washing 4 × 15 min with 500 µL of HCR™ HiFi Probe Wash Buffer at 37 °C.

Amplification

1. Pre-warm HCR™ Gold Amplifier Buffer to room temperature.
2. Remove HCR™ HiFi Probe Wash Buffer from samples.
3. Pre-amplify samples in 500 µL of HCR™ Gold Amplifier Buffer for 30 min at room temperature.
   NOTE: *Use enough HCR™ Gold Amplifier Buffer to immerse sample.*
4. Separately prepare 10 µL of hairpin h1 and 10 µL of hairpin h2. Snap cool each hairpin separately by heating at 95 °C for 90 sec and cooling to room temperature in a dark drawer for 30 min.
5. Prepare amplifier solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to 500 µL of HCR™ Gold Amplification Buffer at room temperature.
6. Remove the pre-amplification solution and add the amplifier solution.

7. Incubate for >3 h at room temperature in the dark.
   NOTE: *Use enough amplifier solution to immerse sample.*
   NOTE: *Increase incubation time for thicker samples (e.g., >12 h for whole-mount vertebrate embryos).*
   NOTE: *For single-molecule imaging, decrease incubation time (e.g., 90 min) if you want single-molecule dots to be diffraction-limited in size.*

8. Pre-warm HCR™ Gold Amplifier Wash Buffer to room temperature.
   NOTE: *HCR™ Gold Amplifier Wash Buffer provided at 4×; dilute to 1× with UltraPure H_2O before use.*

9. Remove excess amplifier by washing 4 × 15 min with 500 µL of HCR™ Gold Amplifier Wash Buffer at room temperature.

10. Store sample at 4 °C and shield from light prior to imaging.

11. Use antifade mounting reagent to mount sample for imaging.
    NOTE: *Use antifade mounting reagent with a counterstain (e.g., DAPI) if preferred.*

Sample Preparation Protocols

This section provides MI-validated sample preparation protocols for:

- FFPE Tissue Sections on a Slide
- Fresh/Fixed Frozen Tissue Sections on a Slide
- Whole-Mount Zebrafish Embryos and Larvae in Solution
- Whole-Mount Mouse Embryos in Solution
- Whole-Mount Chicken Embryos in Solution
- Mammalian Cells on a Chambered Slide

Please note that these sample preparation protocols have not been validated for all sample variations and should only be used as a template.

For more information regarding your specific sample type, please refer to HCR™ RNA-FISH User Examples by Organism and Sample Type, which contains a table of selected user publications that used HCR™ RNA-FISH in diverse organisms and sample types, often including detailed sample preparation protocols. If you do not find your sample type listed, please reach out to our support team for guidance.

If you have previously used another RNA-FISH method on your sample, you can start with the same sample preparation protocol and then perform HCR™ Gold RNA-FISH using a protocol provided above.

Sample Preparation: FFPE Tissue Sections on a Slide

User-Supplied Materials

Reagent†	Supplier	Comments
Pro-Par Clearant	Fisher Scientific	Dewaxing solution
200-Proof Ethanol (EtOH)	Any	—
Target-Retrieval Solution	Any	1× citrate buffer (pH 6.0) or 1× Tris-EDTA buffer (pH 9.0)
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

1. Bake slides in a dry oven for 1 h at 60 °C to improve sample adhesion to the slide.
2. In a fume hood, deparaffinize FFPE tissue by immersing slide in dewaxing solution (e.g., xylene, Pro-Par Clearant etc.) for 3 × 5 min. Move slides up and down occasionally.
   CAUTION: *Dewaxing solution may contain hazardous material, use with care.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Incubate slides in 100% ethanol (EtOH) for 2 × 3 min at room temperature. Move slides up and down occasionally.
4. Rehydrate with a series of graded EtOH washes at room temperature.
   (a) 95% EtOH for 3 min
   (b) 70% EtOH for 3 min
   (c) 50% EtOH for 3 min
   (d) UltraPure H_2O for 3 min
5. Bring 500 mL of 1× Tris-EDTA buffer (pH 9.0) in a beaker to boil in a microwave.
   NOTE: *1× citrate buffer (pH 6.0) can be used in place of Tris-EDTA buffer (pH 9.0).*
6. Maintain Tris-EDTA buffer temperature at 95 °C on a hot plate.
7. Immerse slides for 15 min.
8. Remove beaker from hot plate and add 100 mL of UltraPure H_2O every 5 min to allow temperature to decrease to 45 °C in 20 min.
9. Immerse slides in 400 mL of UltraPure H_2O in a separate container for 10 min at room temperature.
10. Immerse slides in 1× PBST for 2 × 2 min at room temperature.
11. Dry slide using a Kimwipe. Avoid touching the tissue.

12. Draw a barrier around the tissue using a hydrophobic pen.
13. Wash slides by immersing in 1× PBST.
14. Repeat with fresh 1× PBST.
15. Proceed to HCR™ Gold RNA-FISH assay.

Sample Preparation: Fresh/Fixed Frozen Tissue Sections on a Slide

User-Supplied Materials

Reagent†	Supplier	Comments
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
200-Proof Ethanol (EtOH)	Any	—
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
1× PBS	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

1. Remove frozen sections on slides from -80 °C.
2. Fix tissues by immersing slides in ice-cold 4% paraformaldehye (PFA) for 15 min 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.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30 mL is sufficient to immerse sections in the tube. If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Immerse slides in 50% EtOH for 5 min at room temperature.
4. Immerse slides in 70% EtOH for 5 min at room temperature.
5. Immerse slides in 100% EtOH for 5 min at room temperature.
6. Immerse slides in another fresh 100% EtOH for 5 min at room temperature.
7. Immerse slides in 1× PBS.
8. Dry slide using a Kimwipe. Avoid touching the tissue.
9. Draw a barrier around the tissue using a hydrophobic pen.
10. Wash slides by immersing in 1× PBS.
11. Repeat with fresh 1× PBS.
12. Proceed to HCR™ Gold RNA-FISH assay.

Sample Preparation: Whole-Mount Zebrafish Embryos and Larvae in Solution

User-Supplied Materials

Reagent†	Supplier	Comments
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
Egg H_2O	—	See ZFIN General Methods for Zebrafish Care
1-Phenyl 2-thiourea (PTU)	Millipore Sigma	Optional treatment to inhibit pigment development
6% PTU Solution	—	See Common Recipes
0.003% PTU Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes
20 mg/mL Proteinase K	ThermoFisher Scientific	—
30 µg/mL Proteinase K Solution	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

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

1. Collect zebrafish embryos and incubate at 28 °C in a petri dish with egg H_2O.
2. Exchange egg H_2O with 0.003% PTU solution when embryos reach 12 hpf (hours post-fertilization). Replace with fresh 0.003% PTU solution every day until the larvae reach 5 dpf (days post-fertilization).
   NOTE: *Skip this step for embryos that will be imaged younger than 30 hpf as PTU treatment is not necessary.*
   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.*
3. Transfer ∼40 embryos/larvae to a 2 mL eppendorf tube and remove excess egg H_2O.
   NOTE: *Dechorionate embryos that will be imaged younger than 72 hpf before fixation.*
4. 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.*
5. Wash embryos/larvae 3 × 5 min with 1 mL of 1× PBS to stop the fixation.
6. 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.
7. Store embryos/larvae at -20 °C before use.
   NOTE: *Embryos/larvae can be stored for six months at -20 °C.*

8. Transfer the required number of embryos/larvae for an experiment to a 2 mL eppendorf tube.
9. 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.
10. Treat larvae (5 dpf) 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.*
11. Wash embryos/larvae two times with PBST (1 mL each) without incubation.
12. Postfix with 1 mL of 4% PFA for 20 min at room temperature.
13. Wash embryos/larvae 5 × 5 min with 1 mL of PBST.
14. Proceed to HCR™ Gold RNA-FISH assay.

Sample Preparation: Whole-Mount Mouse Embryos in Solution

User-Supplied Materials

Reagent†	Supplier	Comments
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
UltraPure H_2O	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes
20 mg/mL Proteinase K	ThermoFisher Scientific	—
10 µg/mL Proteinase K Solution	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has been optimized for embryos at stage E9.5 and should only be used as a template for other stages.

1. Wipe all dissection equipment with RNaseZap.
2. Kill a pregnant female mouse using an IACUC-approved protocol.
3. Immediately remove the uterus and submerge it in 4% paraformaldehyde (PFA) in a fresh RNase-free petri dish.
   NOTE: *use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
4. Dissect the mouse embryos from the uterus while it is submerged in 4% PFA.
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
   NOTE: *Each female mouse produces 6–9 embryos. We recommend using ≈2 mL of solution per group of 10 embryos.*
5. Transfer the embryos to a clean vial containing fresh 4% PFA and fix them overnight or longer at 4 °C.
   NOTE: *Make sure all embryos are submerged in PFA during fixation.*
6. Wash 2 × 5 min with PBST on ice.
7. Dehydrate embryos into methanol (MeOH) with a series of graded MeOH/PBST washes for 10 min on ice:
   (a) 25% MeOH / 75% PBST
   (b) 50% MeOH / 50% PBST
   (c) 75% MeOH / 25% PBST
   (d) 100% MeOH
   (e) 100% MeOH.

8. Maintain embryos at -20 °C overnight (> 16 h) or until use.
   NOTE: *Embryos can be stored for six months at -20 °C .*
9. Transfer the required number of embryos for an experiment to a 2 mL tube.
   NOTE: *Make sure embryos are submerged during washes.*
10. Rehydrate with a series of graded MeOH/PBST washes for 10 min each on ice:
    (a) 75% MeOH / 25% PBST
    (b) 50% MeOH / 50% PBST
    (c) 25% MeOH / 75% PBST
    (d) 100% PBST
11. Wash embryos with PBST for 10 min at room temperature.
12. Immerse embryos in 10 µg/mL proteinase K solution for 15 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.*
13. Wash embryos 2 × 5 min with PBST.
14. Postfix with 4% PFA for 20 min at room temperature.
    CAUTION: *Use PFA with extreme care as it is a hazardous material.*
15. Wash embryos 3 × 5 min with PBST.
16. Proceed to HCR™ Gold RNA-FISH assay.

Sample Preparation: Whole-Mount Chicken Embryos in Solution

User-Supplied Materials

Reagent†	Supplier	Comments
Ringer's Solution	—	See Common Recipes
Paraformaldehye (PFA)	Millipore Sigma	—
4% Paraformaldehyde (PFA) Solution	—	See Common Recipes
100% Methanol (MeOH)	Any	—
UltraPure H_2O Water	Any	Type I water
10× PBS	Any	Avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
10% Tween-20	Any	—
1× PBST	—	See Common Recipes
20 mg/mL Proteinase K	ThermoFisher Scientific	—
10 µg/mL Proteinase K Solution	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has been optimized for embryos at stage HH 8–11 and should only be used as a template for other stages.

1. Collect chick embryos on 3M paper circles and place in a petri dish containing Ringer's solution.
2. Transfer embryos into a new petri dish with fresh Ringer's solution to rinse away egg yolk before fixation.
3. Transfer into a petri dish containing 4% paraformaldehyde (PFA).
   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.*
4. Fix the samples at room temperature for 1 h.
5. Transfer embryos into a petri dish containing PBST.
6. Dissect the embryos off the filter paper. Remove the fixed vitelline membrane (opaque) and cut the squares around area pellucida, without leaving excess of extra-embryonic tissue.
7. Transfer embryos into a 2 mL tube containing PBST.
8. Nutate for 5 min with the tube positioned horizontally in a small ice bucket.
9. Wash embryos two additional times with 2 mL of PBST, each time nutating for 5 min on ice.
10. Dehydrate embryos with 2 × 5 min washes of 2 mL methanol (MeOH) on ice.
11. Store embryos at -20 °C before use.
    NOTE: *Embryos can be stored for six months at -20 °C.*
12. Transfer the required number of embryos for an experiment to a 2 mL tube.
    NOTE: *do not place more than 4 embryos in each 2 mL tube.*

13. Rehydrate with a series of graded 2 mL MeOH/PBST washes for 5 min each on ice:
    (a) 75% MeOH / 25% PBST
    (b) 50% MeOH / 50% PBST
    (c) 25% MeOH / 75% PBST
    (d) 100% PBST
    (e) 100% PBST.
14. Treat embryos with 2 mL of 10 µg/mL proteinase K solution for 2 min (stage HH 8) or 2.5 min (stage HH 10–11) 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.*
15. Postfix with 2 mL of 4% PFA for 20 min at room temperature.
    CAUTION: *use PFA with extreme care as it is a hazardous material.*
16. Wash embryos 2 × 5 min with 2 mL of PBST on ice.
17. Wash embryos with 2 mL of 50% PBST / 50% 5× SSCT for 5 min on ice.
18. Wash embryos with 2 mL of 5× SSCT for 5 min on ice.
19. Proceed to HCR™ Gold RNA-FISH assay.

Sample Preparation: Mammalian Cells on a Chambered Slide

User-Supplied Materials

Reagent†	Supplier	Comments
DPBS	Gibco	Avoid using DPBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.
UltraPure H_2O	Any	Type I water
Poly-D-Lysine Hydrobromide	Millipore Sigma	—
0.01% (w/v) Poly-D-Lysine	—	See Common Recipes
16% Formaldehyde (FA), Methanol-free	Polysciences	—
4% Formaldehyde (FA) Solution	—	See Common Recipes
200-Proof Ethanol (EtOH)	Any	—
20× SSC	Any	—
2× SSC	—	See Common Recipes

†All user-supplied reagents should be DNase and RNase-free.

Sample Preparation Protocol

This protocol has not been validated for all mammalian cell types and should only be used as a template.

1. Coat bottom of each chamber by applying 300 µL of 0.01% (w/v) poly-D-lysine solution.
   NOTE: *A volume of 300 µL is sufficient per chamber on an 8-chamber slide.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with UltraPure H_2O.
4. Plate desired number of cells in each chamber.
5. Grow cells to desired confluency for 24–48 h.
6. Aspirate growth media and wash each chamber with 300 µL of DPBS.
7. Add 300 µL of 4% formaldehyde to each chamber.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
8. Incubate for 10 min at room temperature.
9. Aspirate fixative and wash each chamber 2 × 300 µL of DPBS.
10. Aspirate DPBS and add 300 µL of ice-cold 70% ethanol.
11. Permeabilize cells overnight at -20 °C.
    NOTE: *Cells can be stored at -20 °C or 4 °C until use.*
12. Prior to in situ hybridization, aspirate EtOH and wash samples 2 × 300 µL of 2× SSC.
13. Proceed to HCR™ Gold RNA-FISH assay.

Common Recipes

NOTE: *avoid using PBS containing calcium chloride or magnesium chloride as these can increase sample autofluorescence.*

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

4% Formaldehyde (FA)
4% formaldehyde
1× PBS

For 10 mL of solution
2.5 mL of 16% formaldehyde
1 mL of 10× PBS
Fill up to 10 mL with UltraPure H_2O

1× PBS
1× PBS

For 50 mL of solution
5 mL of 10× PBS
Fill up to 50 mL with UltraPure H_2O

1× 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

2× SSC
2× sodium chloride sodium citrate (SSC)

For 40 mL of solution
4 mL of 20× SSC
Fill up to 40 mL with UltraPure H_2O

6% PTU Solution
6% PTU

For 100 mL of solution
6 g of 1-phenyl 2-thiourea (PTU) powder
Fill up to 100 mL with egg H_2O
Heat solution at 50–60 °C overnight to dissolve powder

0.003% PTU Solution
0.003% PTU

For 50 mL of solution
25 µL of 6% PTU
Fill up to 50 mL with egg H_2O

Ringer's Solution
123 mM NaCl
1.53 mM $CaCl_2$
4.96 mM KCl_2
0.81 mM Na_2HPO_4
0.15 mM KH_2PO_4

For 2 L of solution
14.4 g of NaCl
340 mg of $CaCl_2$
740 mg of KCl
230 mg of Na_2HPO_4
40 mg of KH_2PO_4
Bring volume up to 1.5 L with UltraPure H_2O
Adjust pH to 7.4 and fill up to 2 L with UltraPure H_2O
Filter sterilize with 0.22 µm bottle top filter

0.01% (w/v) Poly-D-Lysine
0.01% (w/v) Poly-D-Lysine

For 50 mL of solution
5 mg of Poly-D-Lysine Hydrobromide
Fill up to 50 mL with UltraPure H_2O

30 µg/mL 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

10 µg/mL Proteinase K Solution
10 µg/mL proteinase K

For 1 mL of solution
0.5 µL of 20 mg/mL proteinase K
Fill up to 1 mL with PBST

Frequently Asked Questions (FAQ)

I've never used HCR™ Gold RNA-FISH – what's the best way to get started?

- We offer new users a complimentary HCR™ Gold RNA-FISH Starter Kit for performing 3-plex HCR™ Gold RNA-FISH. Please register for an account and fill out this questionnaire.

What comes in an HCR™ Gold RNA-FISH kit?

- HCR™ HiFi Probe
- HCR™ HiFi Probe Hybridization Buffer
- HCR™ HiFi Probe Wash Buffer
- HCR™ Gold Amplifier
- HCR™ Gold Amplifier Buffer
- HCR™ Gold Amplifier Wash Buffer

Can I order a subset of these components?

- Yes. You can order kits containing any subset of the above components.

What HCR™ Gold Amplifiers are available for multiplexing?

- For multiplex experiments, choose a different HCR™ Gold Amplifier (X1, X2, . . . , X10) and label (405, 425, 488, 514, 546, 594, 633, 647, 700, 750, 800) for each target that will be imaged in the same sample (for example, amplifier X3-647 for target 1, amplifier X2-546 for target 2, . . .).
- We recommend labels (488, 546, 647, 750) for robust 4-plex bandpass imaging.
- We recommend labels (405, 425, 488, 514, 546, 594, 633, 700, 750, 800) for robust 10-plex spectral imaging.
- For flexibility, amplifiers X1, X2, and X3 are available with multiple label options (488, 546, 647), while for expanded multiplexing, amplifiers X4–X10 are each available with one label option.
- We recommend using a longer-wavelength label (e.g., 647 or 750) for targets that are more difficult to detect (e.g., low-expression target and/or short target sequence), as autofluorescence tends to be higher in shorter-wavelength channels (e.g., 405 or 488).

What do I order for a multiplex HCR™ Gold RNA-FISH experiment?

- Order one HCR™ Gold RNA-FISH kit for each target RNA:
  - HCR™ HiFi Probe (for use with a different amplifier for each target)
  - HCR™ HiFi Probe Hybridization Buffer
  - HCR™ HiFi Probe Wash Buffer
  - HCR™ Gold Amplifier (for example, amplifier X3 with label 647 for target 1, amplifier X2 with label 546 for target 2, ...)

  - HCR™ Gold Amplifier Buffer
  - HCR™ Gold Amplifier Wash Buffer
  - Note: buffers only needed in one kit

- Example 3-plex experiment:
  - HCR™ Gold RNA-FISH kit for target RNA1:
    - HCR™ HiFi Probe: for target RNA1 for use with amplifier X3
    - HCR™ HiFi Probe Hybridization Buffer (for use with all kits)
    - HCR™ HiFi Probe Wash Buffer (for use with all kits)
    - HCR™ Gold Amplifier: X3 with label 647
    - HCR™ Gold Amplifier Buffer (for use with all kits)
    - HCR™ Gold Amplifier Wash Buffer (for use with all kits)
  - HCR™ Gold RNA-FISH kit for target RNA2:
    - HCR™ HiFi Probe: for target RNA2 for use with amplifier X2
    - HCR™ Gold Amplifier: X2 with label 546
  - HCR™ Gold RNA-FISH kit for target RNA3:
    - HCR™ HiFi Probe: for target RNA3 for use with amplifier X1
    - HCR™ Gold Amplifier: X1 with label 488

Is HCR™ Gold RNA-FISH compatible with clearing techniques?

- Yes, HCR™ Gold RNA-FISH has been shown to be compatible with numerous clearing methods including PACT (Markman *et al.*, 2023), CLARITY (Shah *et al.*, 2016), Ce3D+ (Anderson *et al.*, 2020; Boylan *et al.*, 2020; Lex *et al.*, 2022), TDE (Kim *et al.*, 2023), and SDS (Inagaki *et al.*, 2022; Knoedler *et al.*, 2022; Kozareva *et al.*, 2021). Please contact the MI Team for additional information.

Can I combine HCR™ Gold RNA-FISH with traditional IF for simultaneous RNA and protein imaging?

- Yes, HCR™ Gold RNA-FISH is compatible with traditional immunofluorescence (IF). We recommend performing IF first followed by a 4% paraformaldehyde (PFA) fixation before proceeding to HCR™ Gold RNA-FISH. All IF reagents should be RNase-free.

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).

HCR™ RNA-FISH User Examples by Organism and Sample Type

Organism	Sample types*
African clawed frog	whole-mount embryos (Lee *et al.*, 2023; Leigh *et al.*, 2020; Sun *et al.*, 2022a; Zhao *et al.*, 2022), whole-mount limb and tail (Aztekin *et al.*, 2021)
Amphipod crustacean	whole-mount embryos (Bruce & Patel, 2020, 2022; Bruce *et al.*, 2021)
Ant	whole-mount larvae (Qiu *et al.*, 2022), whole-mount ovaries (Qiu *et al.*, 2022), whole-mount brain (Fetter-Pruneda *et al.*, 2021; Li *et al.*, 2022a; Nagel *et al.*, 2020)
Arabidopsis	whole-mount root (Nobori *et al.*, 2023)
Axolotl	thin tissue sections (Freitas *et al.*, 2019), thin spinal cord tissue section (Duerr *et al.*, 2022), thin lung tissue sections (Jensen *et al.*, 2021), limb buds (Schloissnig *et al.*, 2021; Trofka *et al.*, 2021), thin limb tissue sections (Zhong *et al.*, 2023), thick brain tissue sections (Woych *et al.*, 2022), thin brain tissue sections (Woych *et al.*, 2022)
Bacteria	bacteria on termite gut protozoa (Rosenthal *et al.*, 2013), bacteria in environmental samples (Jia *et al.*, 2021; Yamaguchi *et al.*, 2015), cultured bacteria (Choi *et al.*, 2016; Lassinantti *et al.*, 2021; Needham *et al.*, 2022; Rammohan *et al.*, 2021, 2022), cultured bacterial flow cytometry (Choi *et al.*, 2018; Grieb *et al.*, 2020; Rammohan *et al.*, 2021, 2022), aggregates (Jorth *et al.*, 2019), bacterial symbionts within whole-mount juvenile squid light organ (Bennett *et al.*, 2020), bacteria on cleared plant roots (Dar *et al.*, 2020), bacteria in cleared whole-mount mouse intestines (Gallego-Hernandez *et al.*, 2020), symbionts of the giant tube worms (Hinzke *et al.*, 2021), bacteria in fungal tissue (Dahlstrom & Newman, 2022; Morales *et al.*, 2022; Robinson *et al.*, 2021), bacteria symbionts within sea anemone (Goffredi *et al.*, 2021), bacteria in backskins of mice with clearing (Lay *et al.*, 2018), agar block biofilm (Livingston *et al.*, 2022)
Basal chordate	whole-mount (Kourakis *et al.*, 2019)
Beetle	whole-mount embryos (Bruce & Patel, 2020, 2022; Bruce *et al.*, 2021; Kobayashi *et al.*, 2022; Tidswell *et al.*, 2021), whole-mount heads (Crabtree *et al.*, 2020), whole-mount genitalia (Crabtree *et al.*, 2020), whole-mount ovaries (Luo *et al.*, 2020; Tidswell *et al.*, 2021), thin thoracic tissue sections (Hu *et al.*, 2019), dorsal abdominal segments (Brückner *et al.*, 2021)
Blood fluke	whole-mount (Diaz Soria *et al.*, 2020; Rawlinson *et al.*, 2021)
Brine shrimp	naupili and adults (Bruce & Patel, 2020; Bruce *et al.*, 2021)
Butterfly	whole-mount embryos and imaginal discs (Bruce *et al.*, 2021)
Cavefish	whole-mount embryo (Kozol *et al.*, 2023; O'Gorman *et al.*, 2021)
Chicken	whole-mount embryos (Asmar *et al.*, 2023; Choi *et al.*, 2016, 2018; Galton *et al.*, 2022; Gandhi *et al.*, 2020, 2021; Hutchins *et al.*, 2021; Kim *et al.*, 2022; Ling & Sauka-Spengler, 2019; McLennan *et al.*, 2015; Monroy *et al.*, 2022; Nandagopal *et al.*, 2018; Piacentino & Bronner, 2018; Piacentino *et al.*, 2021; Williams *et al.*, 2019, 2022), thin whole-embryo tissue sections (Askary *et al.*, 2020; Kim *et al.*, 2022), thick cochlea tissue sections (Benkafadar *et al.*, 2021; Janesick *et al.*, 2021), FFPE embryo tissue sections (Rees & Gillis, 2022), utricle tissue sections (Scheibinger *et al.*, 2022)
Daddy long legs	whole-mount embryos (Gainett *et al.*, 2021)
Deep-sea anemone	thin tissue sections (Goffredi *et al.*, 2021)

Organism	Sample types*
Fruit fly	whole-mount embryos (Beaven & Denholm, 2022; Bruce *et al.*, 2021; Choi *et al.*, 2016; Clark *et al.*, 2022b; Domsch *et al.*, 2021; Duk *et al.*, 2021; Graham *et al.*, 2021; Karunaraj *et al.*, 2022; Sankaranarayanan *et al.*, 2021; Sharrock *et al.*, 2022; Surkova *et al.*, 2019; Velten *et al.*, 2022; Zechini *et al.*, 2022), whole-mount pupae (Rose *et al.*, 2022), whole-mount imaginal discs (Bruce *et al.*, 2021; Worley *et al.*, 2022),whole-mount larvae (Ali *et al.*, 2019), whole-mount brains (Lacin *et al.*, 2019; Michki *et al.*, 2021; Sgammeglia *et al.*, 2023; Tang *et al.*, 2022), whole-mount nervous system (Duckhorn *et al.*, 2022a,b), whole-mount ventral nerve cord (Shao *et al.*, 2019), whole-mount ovaries (Dunipace *et al.*, 2022; Lin *et al.*, 2023; Slaidina *et al.*, 2020, 2021; Soriano *et al.*, 2023; Tatapudy *et al.*, 2021; Tu *et al.*, 2021; Zhao *et al.*, 2019), whole-mount testes (Chen *et al.*, 2021b,c), cells (Liu *et al.*, 2023), antenna tissue sections (Task *et al.*, 2022), whole-mount eye-optic lobe (Ali *et al.*, 2019), whole-mount heart (Zechini *et al.*, 2022), whole-mount eye imaginal disc (Kozlov *et al.*, 2022), whole-mount salivary gland (Li *et al.*, 2022b)
Green foxtail	half-mount root (Guillotin *et al.*, 2023)
Hemichordata	whole-mount larva (López *et al.*, 2023)
Honey bee	whole-mount ovary (Cullen *et al.*, 2023)
Human	FFPE thin breast tissue sections (Choi *et al.*, 2016), FFPE thin brain tissue sections (Glineburg *et al.*, 2021), FFPE thin tumor tissue sections (Tanaka *et al.*, 2020), FFPE clear-cell renal-cell carcinoma tissue sections (Kufukihara *et al.*, 2022), cultured cells on a slide (Choi *et al.*, 2018; Fang *et al.*, 2023; Gerbin *et al.*, 2021; Glineburg *et al.*, 2021; Grancharova *et al.*, 2021; Hildebrandt *et al.*, 2023; Kelley *et al.*, 2022; Nandagopal *et al.*, 2019; Pond *et al.*, 2022; Rafiee *et al.*, 2020; Rinaldi *et al.*, 2022; Shilo *et al.*, 2022; Sil *et al.*, 2023; Wen *et al.*, 2021; Zhu *et al.*, 2023), cultured cell flow cytometry (Choi *et al.*, 2018; Gasperini *et al.*, 2019; Reilly *et al.*, 2021), thin brain tissue sections (Fernandez-Cerado *et al.*, 2021; Kamath *et al.*, 2022; Kamermans *et al.*, 2019; Mayerl *et al.*, 2022), thin intestine tissue sections (May-Zhang *et al.*, 2021, 2022), thin kidney tissue sections (Marshall *et al.*, 2022), expanded cultured cells on a slide (Alon *et al.*, 2021), expanded clear-cell renal-cell carcinoma tissue sections (Kufukihara *et al.*, 2022), thin tumor tissue sections and microarrays (Tanaka *et al.*, 2020), organoids (Albanese *et al.*, 2020; Sanaki-Matsumiya *et al.*, 2022; Tanaka *et al.*, 2020; Yamanaka *et al.*, 2022), thin auditory nerve sections (Chen *et al.*, 2022)
Hydra	whole-mount (Vogg *et al.*, 2022)
Killifish	thin coronal sections (van Houcke *et al.*, 2021)
Lancelet	whole-mount embryos (Andrews *et al.*, 2020; Herrera-Úbeda *et al.*, 2019; Zawisza-Álvarez *et al.*, 2020), whole-mount larva (Zawisza-Álvarez *et al.*, 2020), FFPE tissue sections (Sackville *et al.*, 2022), thick tissue sections (Andrews *et al.*, 2020)
Little skate	thin FFPE tissue sections (Criswell & Gillis, 2020; Hirschberger & Gillis, 2022; Marconi *et al.*, 2020; Rees *et al.*, 2023)
Lizard	whole-mount embryo (Sanger *et al.*, 2021)
Maize	half-mount root (Guillotin *et al.*, 2023)
Marmoset	thick brain tissue sections (Krienen *et al.*, 2020), FFPE thin brain tissue sections (Lin *et al.*, 2022)
Minipig	FFPE muscle tissue (Nikovics *et al.*, 2020; Sicherre *et al.*, 2021)
Mosquito	whole-mount embryos (Bui *et al.*, 2023), whole-mount antenna and maxillary palp (Herre *et al.*, 2022; Task *et al.*, 2022), whole-mount ovary (Venkataraman *et al.*, 2023)
Moth	whole-mount pupal wings (Bruce *et al.*, 2021)

Organism	Sample types*
Mouse	whole-mount embryos (Anderson *et al.*, 2020; Boylan *et al.*, 2020; Choi *et al.*, 2016; Huss *et al.*, 2015; Kim *et al.*, 2019; Kumar *et al.*, 2023; Lex *et al.*, 2022; Lohoff *et al.*, 2022; Ramachandran *et al.*, 2022; Trofka *et al.*, 2021; Tyser *et al.*, 2021; Zhu *et al.*, 2022), cleared thick brain tissue sections (Condylis *et al.*, 2022; Greenbaum *et al.*, 2017; Inagaki *et al.*, 2022; Knoedler *et al.*, 2022; Kozareva *et al.*, 2021; Kramer *et al.*, 2018; Mich *et al.*, 2021; Shah *et al.*, 2016; Sylwestrak *et al.*, 2016; Takatoh *et al.*, 2022; Wang *et al.*, 2021), thin brain tissue sections (Askary *et al.*, 2020; Ben-Simon *et al.*, 2022; Carriere *et al.*, 2020; Cleary *et al.*, 2021; Frank *et al.*, 2023; Hu *et al.*, 2022; Ibarra *et al.*, 2021; Kamath *et al.*, 2022; Lui *et al.*, 2021; Mayerl *et al.*, 2021; Osorno *et al.*, 2022; Ren *et al.*, 2019; Shi *et al.*, 2023; Sun *et al.*, 2022b; Young & Song, 2020; Zhang *et al.*, 2022), thin nose tissue sections (Baxter *et al.*, 2020), cultured cells on a slide (Alon *et al.*, 2021; Brookes *et al.*, 2022; Denes *et al.*, 2021; Glineburg *et al.*, 2021; Jain *et al.*, 2022; Nandagopal *et al.*, 2019; Raina *et al.*, 2021; Rodriguez *et al.*, 2020; Shah *et al.*, 2016), expanded thick brain tissue sections (Alon *et al.*, 2021; Arshadi *et al.*, 2021), thick brain tissue sections (Chen *et al.*, 2021d; Hsueh *et al.*, 2023; Kim *et al.*, 2023; Krienen *et al.*, 2020; Michael *et al.*, 2020; Mohan *et al.*, 2023; Norman *et al.*, 2021; Tabata *et al.*, 2022), whole-mount retina (Anderson *et al.*, 2019, 2022), thin spinal cord tissue sections (Li *et al.*, 2020), thin intestine tissue sections (Duque-Correa *et al.*, 2022; May-Zhang *et al.*, 2021), thin retina tissue sections (Zhuang *et al.*, 2020), thin whole-embryo tissue sections (Deal *et al.*, 2021; Liu *et al.*, 2020), thin trigeminal ganglia tissue sections (Emrick *et al.*, 2020; von Buchholtz *et al.*, 2020, 2021), gastruloids (van den Brink *et al.*, 2020; Vianello *et al.*, 2021), expanded thin brain tissue sections (Alon *et al.*, 2021), thin FFPE liver tissue sections (Wells *et al.*, 2021), whole-mount mouse cochlea (Diaz & Heller, 2021), thin cochlear tissue sections (Chen *et al.*, 2022; Ebeid *et al.*, 2022, 2023), thin testes tissue sections (Chen *et al.*, 2021a; Green *et al.*, 2018; Luca *et al.*, 2023), thin kidney tissue sections (Clark *et al.*, 2022a; Marshall *et al.*, 2022), thin digit tissue sections (Johnson *et al.*, 2022), thin tibia tissue sections (Rubin *et al.*, 2021), thin tendon sections (Vinestock *et al.*, 2022), thin optic nerve tissue sections (Wang *et al.*, 2020), thin FFPE digit sections (Castilla-Ibeas *et al.*, 2023), thin FFPE cervical ganglia tissue sections (Ge *et al.*, 2022), thin lymph node tissue section (Koning *et al.*, 2021), thin muscle tissue sections (Pinheiro *et al.*, 2021), whole mount embryo tail (Sanchez & Miyazawa, 2019), whole-mount ganglia (von Buchholtz *et al.*, 2021), thin molar and incisor tissue sections (Nottmeier *et al.*, 2021), whole-mount forelimbs (Markman *et al.*, 2023), whole-mount ear (Jia *et al.*, 2023)
Multi-kingdom	whole-mount juvenile squid light organ and bacterial symbionts (Nikolakakis *et al.*, 2015), consortia of archaea and bacteria (Metcalfe *et al.*, 2021)
Nematode	whole-mount larvae (Belew *et al.*, 2021; Choi *et al.*, 2016; Wong *et al.*, 2018), whole-mount oocyte (Trimmer *et al.*, 2023)
Quail	whole-mount embryo (Huss *et al.*, 2019; Monroy *et al.*, 2022)
Rat	thin rat brain tissue sections (Sui *et al.*, 2016), thin rat femur sections (Nikovics *et al.*, 2022), thick rat brain tissue sections (Chen *et al.*, 2021d)
Rhesus macaque	FFPE thin brain tissue sections (Redmayne & Chavez, 2019)
Sea lamprey	whole-mount embryos (Bedois *et al.*, 2024; Hockman *et al.*, 2019)
Sea urchin	whole-mount embryos (Choi *et al.*, 2016; Ka *et al.*, 2021; Rodríguez-Sastre *et al.*, 2022; Thomas *et al.*, 2023)
Snake	whole-mount embryo (Tzika *et al.*, 2023)
Sorghum	half-mount root (Guillotin *et al.*, 2023)
Sponge	whole-mount sponge (Colgren & Nichols, 2022)
Tarantula	whole-mount embryos (Bruce *et al.*, 2021)
Virus	SARS-CoV-2 in human cells (Kula-Pacurar *et al.*, 2020; Milewska *et al.*, 2020), SARS-Cov2 lateral flow assay (Schulte *et al.*, 2023)
Wasp	whole-mount embryo (Taylor & Dearden, 2022), whole-mount ovaries (Inwood *et al.*, 2023)
Water flea	whole-mount embryos (Bruce & Patel, 2022; Bruce *et al.*, 2021)

Organism	Sample types*
Xenopus	whole-mount embryos (Leigh *et al.*, 2020; Naert *et al.*, 2021; Yoon *et al.*, 2021; Zhao *et al.*, 2022), whole-mount tadpole tails and limbs (Aztekin *et al.*, 2021; Trofka *et al.*, 2021)
Zebrafish	whole-mount embryos (Attardi *et al.*, 2018; Barker *et al.*, 2022; Britto *et al.*, 2022; Bruce *et al.*, 2021; Caviglia *et al.*, 2022; Cayuso *et al.*, 2019; Choi *et al.*, 2010, 2014; Edwards *et al.*, 2023; Fulton *et al.*, 2020; Gallagher *et al.*, 2017; Hageter *et al.*, 2021; Hason *et al.*, 2022; Howard *et al.*, 2021; Ibarra-García-Padilla *et al.*, 2021; Kappel *et al.*, 2022; Kinney *et al.*, 2020; Lencer *et al.*, 2021; Maili *et al.*, 2023; McLaren & Steventon, 2021; Meinecke *et al.*, 2018; Nandamuri *et al.*, 2022; Peloggia *et al.*, 2021; Pond *et al.*, 2021; Rocha *et al.*, 2021; Shah *et al.*, 2016; Sharma *et al.*, 2019; Tao *et al.*, 2021; Thomson *et al.*, 2021; Ton *et al.*, 2018; Trivedi *et al.*, 2018; Truong *et al.*, 2023; Tsai *et al.*, 2020; Wang *et al.*, 2019; Zhao *et al.*, 2022), whole-mount larvae (Basnakova *et al.*, 2021; Bhandiwad *et al.*, 2022; Callahan *et al.*, 2019; Choi *et al.*, 2016; Corradi *et al.*, 2022; Denans *et al.*, 2022; Farrell *et al.*, 2021; Howard *et al.*, 2021, 2022; Ibarra-García-Padilla *et al.*, 2021; Jimenez *et al.*, 2021; Kappel *et al.*, 2022; Lovett-Barron *et al.*, 2017; Martin *et al.*, 2022; Ogawa *et al.*, 2021; Parab *et al.*, 2023; Ruiz *et al.*, 2022; Shainer *et al.*, 2023; Shi *et al.*, 2023; Weinberger *et al.*, 2020; Whitesell *et al.*, 2019; Wurster *et al.*, 2021), embryo tissue sections (Tsai *et al.*, 2020), thin brain tissue sections (O'Brown *et al.*, 2019), FFPE thin heart tissue sections (Simões *et al.*, 2020), whole-mount embryo tails (Thomson *et al.*, 2021), whole-mount heart (Cao *et al.*, 2022; Xia *et al.*, 2022), thin heart tissue sections (Xia *et al.*, 2022), whole-mount ovary (Liu *et al.*, 2022), FFPE ovary tissue sections (Liu *et al.*, 2022)

*Tissue sections are classified as "thick" for thickness $\geq$ 50 µm and "thin" otherwise.

Selected HCR™ RNA-FISH User Publications

Please see this file for the selected user publications cited above.