HCR RNA-FISH/IF for FFPE tisssue sections

This protocol has not been validated for all tissue types and should only be used as a template.
Technical support: support@moleculartechnologies.org

Preparation of formalin-fixed paraffin-embedded (FFPE) tissue sections

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 in Pro-Par Clearant for 3 × 5 min. Move slides up and down occasionally.
   CAUTION: *use Pro-Par Clearant with care as it is a hazardous material.*
   NOTE: *Xylene can be used in place of Pro-Par Clearant.*
   NOTE: *Each 50 mL tube can fit two outward-facing slides. A volume of 30mL 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) Nanopure water for 3 min
5. Bring 500 mL of 1× citrate buffer (pH 6.0) in a beaker to boil in a microwave.
   NOTE: *1× Tris-EDTA buffer (pH 9.0) can be used in place of citrate buffer (pH 6.0). Optimal antigen retrieval method may differ depending on the antigen/antibody used.*
6. Maintain citrate buffer at 90–95 °C on a hot plate.
7. Immerse slides for 15 min.
   NOTE: *Alternatively, slides may be immersed at 95–99 °C for 15 min in a steamer.*
8. Remove beaker from hot plate and add 100 mL of nanopure water every 5 min to allow temperature to decrease to 45 °C in 20 min.
9. Immerse slides in 400 mL of nanopure water in a separate container for 10 min at room temperature.
10. Immerse slides in 1× PBST for 2 × 2 min at room temperature.
    NOTE: *avoid using calcium chloride and magnesium chloride in PBS as this leads to increased autofluorescence in the tissue.*
11. Drain slide by blotting edges on a Kimwipe.
12. Wipe around the section with a Kimwipe and circle tissue with a hydrophobic pen.
13. Optional: Proceed to autofluorescence bleaching protocol if tissue sample has high autofluorescence. Otherwise, proceed to HCR assay.

Buffer recipes for sample preparation

1× citrate buffer	For 500 mL of solution
1× citrate buffer	5 mL of 100× citrate buffer (pH 6.0) Fill up to 500 mL with water

1× Tris-EDTA buffer	For 500 mL of solution
1× Tris-EDTA buffer	5 mL of 100× Tris-EDTA buffer (pH 9.0) Fill up to 500 mL with water

PBST	For 50 mL of solution
1× PBS 0.1% Tween 20	5 mL of 10× PBS 500 μL of 10% Tween 20 Fill up to 50 mL with ultrapure H_2O

Optional autofluorescence bleaching protocol

1. Prepare bleaching solution fresh before use.
   CAUTION: *Keep bleaching solution uncapped inside a fume hood as it produces gas.*
2. Add 200 μL of bleaching solution on top of tissue.
3. Place slide under a 240 W LED light. Keep slide 80 mm away from the light source.
   NOTE: *Perform bleaching inside a refrigerator to avoid overheating of sample.*
4. Expose tissue to maximum LED intensity for 3 h.
   NOTE: *Check slide every hour and re-apply fresh bleaching solution if necessary.*
5. Wash slide 4 × 10 min in PBST.
6. Proceed to HCR assay.

Buffer recipes for autofluorescence bleaching protocol

Bleaching solution	For 1 mL of solution
4.5% hydrogen peroxide (H_2O_2) 24 mM NaOH 1× PBS	150 μL 30% H_2O_2 4.8 μL of 5 M NaOH 845.2 μL 1× PBS

Multiplexed HCR IF with/without HCR RNA-FISH

Unlabeled primary antibody probes and initiator-labeled secondary antibody probes for protein targets, split-initiator DNA probes for RNA targets, and simultaneous HCR signal amplification for all targets.

Protein detection stage

1. Block tissue by applying 200 μL of antibody buffer on top of the sample. Incubate at room temperature for 1 h in a humidified chamber.
2. Prepare working concentration of primary antibodies in antibody buffer. Prepare 100 μL per section.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
3. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
4. Add primary antibody solution to each section and incubate overnight (>12 h) at 4 °C in a humidified chamber.
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on sample type and thickness.*
5. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
6. Prepare 1μg/mL working concentration of initiator-labeled secondary antibodies in antibody buffer. Prepare 100 μL per section.
7. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
8. Add secondary antibody solution to each section and incubate for 1 h at room temperature in a humidified chamber.
9. Remove excess antibodies by immersing slide in 1× PBST at room temperature for 3 × 5 min.
10. Proceed to **RNA detection stage** for co-detection of protein and RNA. Otherwise, proceed to **Amplification stage**.

RNA detection stage

1. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
2. Post-fix sample by adding 200 μL of 4% formaldehyde on the tissue.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
3. Incubate slides for 10 min at room temperature.
4. Immerse slides for 2 × 5 min in PBST.
5. Immerse slides for 5 min in 5× SSCT.
6. Pre-warm a humidified chamber to 37 °C.
7. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
8. Add 200 μL of probe hybridization buffer on top of the tissue sample.
   CAUTION: *Probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe hybridization buffer to 37 °C before use.*
9. Pre-hybridize for 10 min inside the humidified chamber.

10. Prepare a 16 nM probe solution by adding 1.6 pmol of each probe set (e.g. 1.6 μL of 1 μM stock) to 100 μL of probe hybridization buffer at 37 °C.
    NOTE: *This is the amount of probe set needed for each target on a single slide using 100 μL of incubation volume.*

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

12. Add 100 μL of the probe solution on top of the tissue sample.

13. Place a coverslip on the sample and incubate overnight (>12 h) in the 37 °C humidified chamber.

14. Immerse slide in probe wash buffer at 37 °C to float off coverslip.
    CAUTION: *Probe wash buffer contains formamide, a hazardous material.*

15. Remove excess probes by incubating slide at 37 °C in:

    (a) 75% of probe wash buffer / 25% 5× SSCT for 15 min
    (b) 50% of probe wash buffer / 50% 5× SSCT for 15 min
    (c) 25% of probe wash buffer / 75% 5× SSCT for 15 min
    (d) 100% 5× SSCT for 15 min

    NOTE: *Wash solutions should be pre-heated to 37 °C before use.*

16. Proceed to **Amplification stage**.

Amplification stage

1. Immerse slide in 5× SSCT at room temperature for 5 min.
2. Drain slide by blotting edges on a Kimwipe and wipe around the section with another Kimwipe.
3. Add 200 μL of amplification buffer on top of the tissue sample and pre-amplify in a humidified chamber for 30 min at room temperature.
   NOTE: *equilibrate amplification buffer to room temperature before use.*
4. 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. This is the amount of hairpins needed for each target on a single slide using 100 μL of incubation volume.*
5. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 100 μL of amplification buffer at room temperature per section.
6. Remove the pre-amplification solution and drain excess buffer on slide by blotting edges on a Kimwipe.
7. Add 100 μL of the hairpin solution on top of the tissue sample.
8. Incubate overnight (>12 h) in a dark humidified chamber at room temperature.
9. Remove excess hairpins by immersing slide in 5× SSCT at room temperature for:
   (a) 1 × 5 min
   (b) 2 × 15 min
   (c) 1 × 5 min

Sample mounting for microscopy

1. Drain slide by blotting edges on a Kimwipe and dry around the section with another Kimwipe.
2. Apply 50-100 μL of antifade mountant on top of the sample.
3. Place a 22 × 30 mm No. 1 coverslip on top carefully to prevent air bubbles.
4. Slides can be stored at 4 °C protected from light prior to imaging.

Buffers for HCR IF with/without HCR RNA-FISH

HCR probes (initiator-labeled antibody probes, split-initiator DNA probes), amplifiers, and buffers (antibody buffer, probe hybridization buffer, probe wash buffer, amplification buffer) are available from Molecular Technologies (www.moleculartechnologies.org). Probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Antibody buffer and amplification buffer should be stored at 4 °C. Make sure all solutions are well mixed before use.

5× SSCT	For 40 mL of solution
5× saline sodium citrate (SSC)	10 mL of 20× SSC
0.1% Tween 20	400 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O

Reagents and supplies

Pro-Par Clearant (ANATECH LTD Cat. # 510)
100% Ethanol (EtOH) (VWR Cat. # 89125-172)
100× citrate buffer pH 6.0 (Abcam Cat. # ab93678)
100× Tris-EDTA buffer pH 9.0 (Abcam Cat. # ab93684)
10× Phosphate-buffered saline (PBS) (Invitrogen Cat. # AM9624)
30% hydrogen peroxide (Sigma Aldrich Cat. #H1009)
Sodium hydroxide (Fisher Scientific Cat. #S318-500)
20× saline sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
10% Tween 20 (Teknova Cat. # T0710)
SlowFade Diamond Antifade Mountant with DAPI (Invitrogen Cat. # S36973)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
6 band 240 W LED vegetative grow light (HTG Supply Cat. # LED-6B240)

Citation Notes

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- **10-plex HCR spectral imaging**
  HCR spectral imaging of any combination of 10 RNA and/or protein targets with 1-step quantitative HCR signal amplification for all targets simultaneously (Schulte *et al.*, 2024a).
  Software: HCR Imaging Python module including Dot Detection 2.0 and Unmix 1.0 packages.

- **HCR protein:protein complex imaging**
  Multiplexed, quantitative, high-resolution imaging of protein:protein complexes with/without HCR IF and/or HCR RNA-FISH with simultaneous HCR signal amplification for all protein:protein, protein, and RNA targets (Schulte *et al.*, 2024b).

- **HCR RNA-FISH/IF**
  A unified approach to multiplexed, quantitative, high-resolution RNA fluorescence in situ hybridization (RNA-FISH) and protein immunofluorescence (IF), with quantitative 1-step enzyme-free HCR signal amplification performed for all RNA and protein targets simultaneously (Schwarzkopf *et al.*, 2021).

- **HCR IF**
  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)**
  Automatic background suppression for dramatically enhanced performance (signal-to-background and quantitative precision) and ease-of-use (no probe set optimization for new targets) (Choi *et al.*, 2018). v3.0 supports three quantitative modes:

  qHCR imaging: analog mRNA relative quantitation with subcellular resolution in an anatomical context;

  qHCR flow cytometry: analog mRNA relative quantitation for high-throughput single-cell analysis;

  dHCR imaging: digital mRNA absolute quantitation with single-molecule resolution in an anatomical context.

  Protocols for v3.0 in diverse organisms are adapted from the zoo paper.
  Software: Dot Analysis 1.0 package.

- **Quantitative HCR (qHCR) imaging with subcellular resolution**
  Analog mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos. The read-out/read-in analysis framework enables bidirectional quantitative discovery in an anatomical context (Trivedi *et al.*, 2018).
  Software: Read-out/Read-in 1.0 package.

- **Zoo paper**
  Protocols for multiplexed mRNA imaging in diverse sample types (Choi *et al.*, 2016):

bacteria in suspension	whole-mount zebrafish embryos and larvae
whole-mount fruit fly embryos	whole-mount chicken embryos
whole-mount worm larvae	whole-mount mouse embryos
whole-mount sea urchin embryos	FFPE human tissue sections

- **Digitial HCR (dHCR) imaging with single-molecule resolution**
  Digital mRNA absolute quantitation with single-molecule resolution in thick autofluorescent samples (e.g., 0.5 mm adult mouse brain sections) (Shah *et al.*, 2016).

- **Multiplexed quantitative HCR (qHCR) northern blots**
  Simultaneous quantification of RNA target size and abundance with signal amplification for up to 5 target RNAs (Schwarzkopf & Pierce, 2016).

- **HCR RNA-FISH (v2.0)**
  2nd generation HCR RNA-FISH technology (v2.0) using DNA 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)**
  1st generation HCR RNA-FISH technology (v1.0) using RNA 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 isothermal enzyme-free molecular signal amplification (Dirks & Pierce, 2004).

We are happy to provide advice and technical support: support@moleculartechnologies.org.

– The Molecular Technologies Team

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