HCR protein:protein complex imaging in FFPE tissue sections

This protocol has not been validated for all FFPE 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 the slide in Pro-Par Clearant for 2 × 5 min. Gently move the slide up and down every minute.
   NOTE: *If desired, a larger number of slides can be processed together using a Coplin jar.*
3. Immerse the slide in 100% ethanol (EtOH) for 2 × 3 min at room temperature. Gently move the slide up and down every minute.
4. Immerse the slide in 95% EtOH for 3 min at room temperature.
5. Immerse the slide in 70% EtOH for 3 min at room temperature.
6. Immerse the slide in nanopure H_2O for 3 min at room temperature.
7. Heat antigen retrieval buffer in a heatproof container with a digital steamer until >98 °C.
8. Immerse the slide in the heated antigen retrieval buffer in the digital steamer for 15 min.
9. Remove the slide from the antigen retrieval buffer and immediately immerse in nanopure H_2O for 10 min at room temperature.
10. Remove the slide and gently tap off excess nanopure H_2O.
11. Carefully dry around the tissue with a Kimwipe.
12. Draw a hydrophobic barrier around the tissue with a hydrophobic pen.
13. Place the slide in a humidified chamber.
    NOTE: *Keep the slide in a humidified chamber for all future steps to prevent evaporation.*

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Protein detection stage

1. Apply antibody buffer to the tissue section. Incubate for 1 h at room temperature.
   NOTE: *Scale volumes according to the size of the tissue. A volume of 100 µL was utilized here for each step.*
2. Prepare working concentration of primary antibodies in antibody buffer.
   NOTE: *Follow the manufacturer's guidelines for the primary antibody working concentration.*
3. Replace antibody buffer with primary antibody solution and incubate overnight (>12 h) at 4 °C.
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on the antibody used.*
4. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature.
5. Prepare secondary antibody probe solution containing split-initiator secondary antibody probes (p1 and p2) at 1 µg/mL in antibody buffer.
6. Add secondary antibody probe solution to the sample. Incubate for 1 h at room temperature.
7. Remove excess secondary antibody probes by washing 3 × 5 min with 1× PBST at room temperature.
8. Wash with 5× SSCT for 5 min at room temperature.
9. Add amplification buffer. Incubate for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
10. Prepare a 1.6 nM proximity probe solution in amplification buffer at room temperature.
11. Remove amplification buffer and add proximity probe solution. Incubate for 1 h at room temperature.
12. Wash 3 × 5 min with 5× SSCT at room temperature.

Amplification stage

1. Add amplification buffer. Incubate for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*
2. Separately prepare hairpin h1 and hairpin h2 by snap cooling (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 a 60 nM hairpin solution by adding snap-cooled h1 hairpins and snap-cooled h2 hairpins to amplification buffer at room temperature.
4. Remove the amplification buffer and add the hairpin solution.
5. Incubate overnight (>12 h) protected from light at room temperature.
6. Remove excess hairpins by washing with 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 15 min
   (c) 1 × 5 min

Sample mounting for microscopy

1. Carefully dry around the section with a Kimwipe.
2. Apply 60 μL of DAPI Fluoromount-G to the section.
3. Carefully lower a 22 mm $\times$ 30 mm No. 1.5 coverslip on top of the section.
4. Slides can be stored at 4 °C protected from light prior to imaging.

Buffers for HCR protein:protein complex imaging in FFPE tissue sections

HCR probes, amplifiers, and buffers (antibody buffer and amplification buffer) are available from Molecular Technologies. Antibody buffer and amplification buffer should be stored at 4 °C. Make sure all solutions are well mixed before use.

PBST	For 500 mL of solution
1$\times$ PBS	50 mL of 10$\times$ PBS
0.1% Tween-20	5 mL of 10% Tween-20
	Fill up to 500 mL with ultrapure H_2O
	Filter with a 0.2 μm Nalgene Rapid-Flow filter

5$\times$ SSCT	For 500 mL of solution
5$\times$ saline sodium citrate (SSC)	125 mL of 20$\times$ SSC
0.1% Tween-20	5 mL of 10% Tween-20
	Fill up to 500 mL with ultrapure H_2O
	Filter with a 0.2 μm Nalgene Rapid-Flow filter

Reagents and supplies

Pro-Par Clearant (ANATECH LTD, 510)
100% EtOH (Koptec, V1001)
Antigen retrieval buffer (100X Citrate Buffer) (Abcam, ab93678)
10$\times$ PBS (Invitrogen, AM9624)
20$\times$ Saline sodium citrate (SSC) (Invitrogen, 15557-044)
10% Tween-20 (Teknova, T0710)
DAPI Fluoromount-G (SouthernBiotech, 0100-20)

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

References

Choi, H. M. T., Chang, J. Y., Trinh, L. A., Padilla, J. E., Fraser, S. E., & Pierce, N. A. (2010). Programmable in situ amplification for multiplexed imaging of mRNA expression. *Nat Biotechnol*, **28**(11), 1208–12. (pdf, supp info, supp movie 1, supp movie 2)

Choi, H. M. T., Beck, V. A., & Pierce, N. A. (2014). Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. *ACS Nano*, **8**(5), 4284–4294. (pdf, supp info, supp movie)

Choi, H. M. T., Calvert, C. R., Husain, N., Huss, D., Barsi, J. C., Deverman, B. E., Hunter, R. C., Kato, M., Lee, S. M., Abelin, A. C. T., Rosenthal, A. Z., Akbari, O. S., Li, Y., Hay, B. A., Sternberg, P. W., Patterson, P. H., Davidson, E. H., Mazmanian, S. K., Prober, D. A., van de Rijn, M., Leadbetter, J. R., Newman, D. K., Readhead, C., Bronner, M. E., Wold, B., Lansford, R., Sauka-Spengler, T., Fraser, S. E., & Pierce, N. A. (2016). Mapping a multiplexed zoo of mRNA expression. *Development*, **143**, 3632–3637. (pdf, supp info, fruit fly movie, sea urchin movie, zebrafish movie, chicken movie, mouse movie)

Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development*, **145**, dev165753. (pdf, supp info, Dot Analysis 1.0 package)

Dirks, R. M., & Pierce, N. A. (2004). Triggered amplification by hybridization chain reaction. *Proc Natl Acad Sci USA*, **101**(43), 15275–15278. (pdf)

Schulte, S.J., Fornace, M.E., Hall, J.K., Shin, G.J., & Pierce, N.A. (2024a). HCR spectral imaging: 10-plex, quantitative, high-resolution RNA and protein imaging in highly autofluorescent samples. *Development*, **151**(4), dev202307. (pdf, supp info, HCR Imaging Python Module containing Dot Detection 2.0 and Unmix 1.0 packages)

Schulte, S.J., Shin, B., Rothenberg, E.V., & Pierce, N.A. (2024b). Multiplex, quantitative, high-resolution imaging of protein:protein complexes via hybridization chain reaction. *ACS Chem Biol*, **19**(2), 280–288. (pdf, supp info)

Schwarzkopf, M., & Pierce, N. A. (2016). Multiplexed miRNA northern blots via hybridization chain reaction. *Nucleic Acids Res*, **44**(15), e129. (pdf, supp info)

Schwarzkopf, M., Liu, M. C., Schulte, S. J., Ives, R., Husain, N., Choi, H. M. T., & Pierce, N. A. (2021). Hybridization chain reaction enables a unified approach to multiplexed, quantitative, high-resolution immunohistochemistry and in situ hybridization. *Development*, **148**(22), dev199847. (pdf, supp info)

Shah, S., Lubeck, E., Schwarzkopf, M., He, T.-F., Greenbaum, A., Sohn, C. H., Lignell, A., Choi, H. M. T., Gradinaru, V., Pierce, N. A., & Cai, L. (2016). Single-molecule RNA detection at depth via hybridization chain reaction and tissue hydrogel embedding and clearing. *Development*, **143**, 2862–2867. (pdf, supp info, supp movie 1, supp movie 2, supp movie 3)

Trivedi, V., Choi, H. M. T., Fraser, S. E., & Pierce, N. A. (2018). Multidimensional quantitative analysis of mRNA expression within intact vertebrate embryos. *Development*, **145**, dev156869. (pdf, supp info, Read-out/Read-in 1.0 package)