HCR IF/RNA-FISH for mammalian cells on a chambered slide

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

Preparation of fixed mammalian cells on a chambered slide

1. Coat bottom of each chamber by applying 300 μL of 0.01% poly-D-lysine prepared in cell culture grade H_2O.
   NOTE: *A volume of 300 μL is sufficient per chamber on an 8-chamber slide. Scale volume accordingly if using a different slide format.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber twice with molecular biology grade 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.
   NOTE: *avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
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. Remove fixative and wash each chamber with 2×300 μL of DPBS.
10. Aspirate DPBS and add 300 μL of ice-cold 70% ethanol (EtOH).
11. Permeabilize cells overnight (or longer) at -20 °C.
12. Proceed to HCR assay.

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. Aspirate EtOH from sample and wash samples 2 × 5 min with 300 μL of 1× PBS.
2. Apply 300 μL antibody buffer to each chamber. Incubate at room temperature for 1 hr with gentle agitation.
3. Prepare working concentration of primary antibodies in antibody buffer. Prepare 300 μL per chamber.
   NOTE: *follow manufacturer's guidelines for primary antibody working concentration.*
4. Replace antibody buffer with primary antibody solution and incubate overnight (>12 h) at 4 °C with gentle agitation.
   NOTE: *Incubation may be optimized (e.g., 1–2 h at room temperature) depending on sample type and thickness.*
5. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
6. Prepare 1 μg/mL working concentration of initiator-labeled secondary antibodies in antibody buffer. Prepare 300 μL per chamber.
   NOTE: *Concentration may be optimized depending on protein target and primary antibody.*
7. Add secondary antibody solution to each chamber and incubate 1 h at room temperature with gentle agitation.
8. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
9. Proceed to **RNA detection stage** for co-detection of protein and RNA. Otherwise, proceed to **Amplification stage**.

RNA detection stage

1. Post-fix sample with 300 μL of 4% formaldehyde.
   CAUTION: *use formaldehyde with extreme care as it is a hazardous material.*
2. Incubate for 10 min at room temperature.
3. Remove fixative and wash each chamber with 2 × 300 μL of PBS.
4. Wash sample with 300 μL of 2× SSC.
5. Pre-hybridize samples in 300 μL of probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *Probe hybridization buffer contains formamide, a hazardous material.*
   NOTE: *pre-heat probe hybridization buffer to 37 °C before use.*
6. Prepare a 16 nM probe solution by adding 4.8 pmol of each probe mixture (e.g. 4.8 μL of 1 μM stock) to 300 μL of probe hybridization buffer at 37 °C.
   NOTE: *This is the amount of probe set needed for each target on a single chamber of an 8-well chambered slide using 300 μL of incubation volume.*
7. Remove the pre-hybridization solution and add the probe solution.
8. Incubate samples overnight (>12 h) at 37 °C.

9. Remove excess probes by washing 4 × 5 min with 300 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.*

10. Wash with 300 μL 5× SSCT at room temperature for 5 min.

11. Proceed to **Amplification stage**.

Amplification stage

1. Wash with 300 μL 5× SSCT at room temperature for 5 min.

2. Pre-amplify samples in 300 μL of amplification buffer for 30 min at room temperature.
   NOTE: *Equilibrate amplification buffer to room temperature before use.*

3. Separately prepare 18 pmol of hairpin h1 and 18 pmol of hairpin h2 by snap cooling 6 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 in a single sample using 300 μL of incubation volume.*

4. Prepare a 60 nM hairpin solution by adding all snap-cooled h1 hairpins and snap-cooled h2 hairpins to 300 μL of amplification buffer at room temperature per sample.

5. Remove the pre-amplification solution and add the hairpin solution.

6. Incubate the slide overnight ($>$12 h) protected from light at room temperature.

7. Remove excess hairpins by washing 5 × 5 min with 300 μL of 5× SSCT at room temperature.

Sample mounting for microscopy

1. Remove final wash and add 150 μL of mounting medium (e.g., Fluoromount-G with DAPI).

2. 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.

1× PBST	For 40 mL of solution
1× phosphate-buffered saline (PBS)	4 mL of 10× PBS
0.1% Tween 20	400 μL of 10% Tween 20
	Fill up to 40 mL with ultrapure H_2O

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

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

ibidi μ-slide ibitreat (ibidi Cat. # 80826)
Poly-D-lysine hydrobromide (Sigma-Aldrich Cat. # P7280)
Molecular biology grade H_2O (Corning Cat. # 46-000-CV)
DPBS, no calcium, no magnesium (Life Technologies Cat. # 14190144)
Image-iT Fixative Solution 4% (Thermo Fisher Scientific Cat. # FB002)
10× PBS (Ambion Cat. # AM9624)
10% Tween 20 (Teknova Cat. # T0710)
20× saline sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
DAPI Fluoromount-G (SouthernBiotech Cat. # 0100-20)

Citation Notes

Molecular Technologies (moleculartechnologies.org) is a non-profit academic resource within the Beckman Institute at Caltech. For citation, please select from the list below as appropriate for your application:

- **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 fruit fly embryos
  whole-mount worm larvae
  whole-mount sea urchin embryos
  whole-mount zebrafish embryos and larvae
  whole-mount chicken embryos
  whole-mount mouse 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)