Molecular Technologies
MAMMALIAN CELLS ON A SLIDE

HCR protein:protein complex imaging 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 adherent mammalian cells on a chambered slide

1. Coat the bottom of each chamber by applying 100 μL of 0.01% poly-D-lysine prepared in molecular biology grade H_2O.
   NOTE: *For each step, a volume of 100 μL is sufficient per chamber on an 18-chamber slide. Scale volumes accordingly if using a different chambered format.*
2. Incubate for at least 30 min at room temperature.
3. Aspirate the coating solution and wash each chamber 2$\times$ with molecular biology grade H_2O.
4. Add the desired number of cells to each chamber.
5. Grow the cells to the desired confluency for 24–48 h.
6. Aspirate the growth media and wash each chamber with DPBS.
   NOTE: *Avoid using DPBS with calcium chloride and magnesium chloride, as this leads to increased autofluorescence.*
7. Add 4% formaldehyde in PBS 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 2$\times$ with DPBS.
10. Aspirate DPBS and add ice-cold 70% ethanol (EtOH) to permeabilize the cells.
11. Permeabilize cells for 3 h at 4 °C.
    NOTE: *Alternatively, the cells may be permeabilized overnight (or longer) at -20 °C.*

HCR protein:protein complex imaging with/without HCR IF and HCR RNA-FISH using simultaneous HCR signal amplification for all protein:protein, protein, and RNA targets

Protein detection stage

1. Aspirate EtOH from sample and wash 2 × 5 min with 1× PBS.
2. Add antibody buffer to the sample. Incubate for 1 h at room temperature with gentle agitation.
3. Prepare working concentration of primary antibodies in antibody buffer.
   NOTE: *Follow the manufacturer's guidelines for the 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 the antibody used.*
5. Remove excess antibodies by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
6. Prepare secondary antibody probe solution containing split-initiator secondary antibody probes (p1 and p2), and optionally initiator-labeled secondary antibodies for HCR IF, at 1 μg/mL in antibody buffer.
7. Add the secondary antibody probe solution to the sample. Incubate for 1 h at room temperature with gentle agitation.
8. Remove excess secondary antibody probes by washing 3 × 5 min with 1× PBST at room temperature with gentle agitation.
9. Wash with 5× SSCT for 5 min at room temperature.
10. Add amplification buffer. Incubate for 30 min at room temperature.
    NOTE: *Equilibrate amplification buffer to room temperature before use.*
11. Prepare a 1.6 nM proximity probe solution in amplification buffer at room temperature.
12. Remove amplification buffer and add proximity probe solution. Incubate for 1 h at room temperature.
13. Wash 3 × 5 min with 5× SSCT at room temperature.
14. Proceed to **RNA detection stage** for co-detection of RNA. Otherwise, proceed to **Amplification stage**.

RNA detection stage

1. Post-fix sample with 4% formaldehyde in PBS. Incubate for 10 min at room temperature.
   CAUTION: *Use formaldehyde with extreme care, as it is a hazardous material.*
2. Wash 3 × 5 min with 1× PBST at room temperature.
3. Wash with 5× SSCT for 5 min at room temperature.
4. Add probe hybridization buffer. Incubate 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.*
5. Prepare a 16 nM probe solution in probe hybridization buffer at 37 °C.
6. Remove the probe hybridization buffer and add the probe solution.
7. Incubate overnight (>12 h) at 37 °C.

8. Remove excess probes by washing 4 × 5 min with 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.*
9. Wash 3 × 5 min with 5× SSCT at room temperature.
10. Proceed to **Amplification stage**.

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 5 × 5 min with 5× SSCT at room temperature.

Sample mounting for microscopy

1. Add DAPI Fluoromount-G mounting medium.
2. The coverslip can be stored at 4 °C protected from light prior to imaging.

Buffers for HCR protein:protein complex imaging with/without HCR IF and HCR RNA-FISH

HCR probes (split-initiator antibody 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 (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.

PBST	For 500 mL of solution
1× PBS	50 mL of 10× 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× SSCT	For 500 mL of solution
5× saline sodium citrate (SSC)	125 mL of 20× 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

ibidi μ-slide 18 well ibiTreat (ibidi, 81816)
Poly-D-lysine hydrobromide (Sigma-Aldrich, P7280)
Molecular biology grade H_2O (Corning, 46-000-CV)
DPBS, no calcium, no magnesium (Gibco, 14190144)
Image-iT 4% formaldehyde fixative solution in PBS (methanol-free) (Invitrogen, FB002)
100% EtOH (Koptec, V1001)
10× PBS (Invitrogen, AM9624)
20× 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

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