Molecular Technologies
MAMMALIAN CELLS IN SUSPENSION

HCR RNA-FISH v3.0 protocol for mammalian cells in suspension

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

Sample preparation protocol

1. Aspirate growth media from culture plate and wash cells with DPBS.
   NOTE: *Avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
2. Add 3 mL of trypsin per 10 cm plate and incubate in a 5% CO_2 incubator at 37 °C for 5 min.
3. Quench trypsin by adding 3 mL of growth media.
4. Transfer cells to a 15 mL conical tube and centrifuge for 5 min at 180 × g.
5. Aspirate supernatant and re-suspend cells in 4% formaldehyde to reach approximately 10^6 cells/mL.
   CAUTION: *Use formaldehyde with extreme care as it is a hazardous material.*
6. Fix cells for at least 1 hr at room temperature.
7. Centrifuge for 5 min at 180 × g and aspirate supernatant.
8. Wash cells 4 times with PBST (use the same volume as formaldehyde solution).
9. Re-suspend cells in cold 70% ethanol (use the same volume as formaldehyde and PBST solutions).
10. Store cells at 4 °C overnight before use.

Buffer recipes for sample preparation

4% formaldehyde in PBST

4% formaldehyde
1× PBS
0.1% Tween 20

For 10 mL of solution
2.5 mL of 16% formaldehyde
1 mL of 10× PBS
100 μL of 10% Tween 20
Fill up to 10 mL with ultrapure H_2O

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

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

Multiplexed HCR RNA-FISH v3.0 protocol

Detection stage

1. Transfer desired amount (0.5-1 $\times$ 10^6) of fixed cells into a 1.5 mL eppendorf tube.
2. Centrifuge for 5 min and remove supernatant.
   NOTE: *Centrifugation should be as gentle as possible to pellet cells. All centrifugation steps are done at 180 $\times$ g.*
3. Wash cells twice with 500 μL of PBST. Centrifuge for 5 min to remove supernatant.
4. Re-suspend the pellet with 400 μL of 30% probe hybridization buffer and pre-hybridize for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
5. In the meantime, prepare probe solution by adding 2 pmol of each probe mixture (e.g. 2 μL of 1 μM stock) to 100 μL of 30% probe hybridization buffer pre-heated to 37 °C.
6. Add the probe solution directly to the sample to reach a final probe concentration of 4 nM.
   NOTE: *For single-molecule RNA imaging, use higher probe concentration (e.g., 16 nM) to increase probe hybridization yield. If desired, this approach can also be used to increase signal for subcellular quantitative RNA imaging.*
7. Incubate the sample overnight at 37 °C.
8. Centrifuge for 5 min to remove probe solution.
9. Re-suspend the cell pellet with 500 μL of 30% probe wash buffer.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Wash solutions should be pre-heated to 37°C before use.*
10. Incubate for 10 min at 37 °C and remove the wash solution by centrifugation for 5 min.
11. Repeat steps 9 and 10 for three additional times.
12. Re-suspend the cell pellet with 500 μL of 5$\times$ SSCT.
13. Incubate for 5 min at room temperature.
14. Proceed to hairpin amplification.

Amplification stage

1. Centrifuge for 5 min to pellet the cells.
2. Re-suspend the cell pellet with 150 μL of amplification buffer and pre-amplify for 30 min at room temperature.
3. Separately prepare 15 pmol of hairpin H1 and 15 pmol of hairpin H2 by snap cooling 5 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.*
4. Prepare hairpin mixture by adding all snap-cooled hairpins to 100 μL of amplification buffer at room temperature.
5. Add the hairpin mixture directly to the sample to reach a final hairpin concentration of 60 nM.

6. Incubate the sample overnight (>12 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for 45-min to ensure single-molecule dots are diffraction-limited.*
7. Centrifuge for 5 min and remove the hairpin solution.
8. Re-suspend the cell pellet with 500 μL of 5$\times$ SSCT.
9. Without incubation, remove the wash solution by centrifugation for 5 min.
10. Repeat steps 8 and 9 for five additional times.
11. Re-suspend the cell pellet in desired buffer and volume.
12. Filter cells before flow cytometry.

HCR reagents storage conditions

Probes, amplifiers, probe hybridization buffer, and probe wash buffer should be stored at -20 °C. Amplification buffer should be stored at 4 °C. Keep these reagents on ice at all times during probe and amplifier preparation. Make sure all solutions are well mixed before use.

General buffer recipes for HCR RNA-FISH

5× SSCT	For 40 mL of solution
5× sodium chloride 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

DPBS, no calcium, no magnesium (Life Technologies Cat. # 14190144)
Trypsin-EDTA (0.25%), phenol red (Life Technologies Cat. # 25200072)
16% Formaldehyde (w/v), methanol-free (Life Technologies Cat. # 28908)
10× PBS (Life Technologies Cat.# AM9625)
10% Tween 20 solution (Bio-Rad Cat. # 161-0781)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)

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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