# Protocols 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 $\mu$L of 0.01% poly-D-lysine prepared in cell culture grade $H_2O$.
   NOTE: *A volume of 300 $\mu$L is sufficient per chamber on an 8-chamber slide.*
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 $\mu$L of DPBS.
   NOTE: *Avoid using calcium chloride and magnesium chloride in DPBS as this leads to increased autofluorescence.*
7. Add 300 $\mu$L 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. Aspirate fixative and wash each chamber 2 $\times$ 300 $\mu$L of DPBS.
10. Aspirate DPBS and add 300 $\mu$L of ice-cold 70% ethanol.
11. Permeabilize cells overnight at -20 °C.
12. Cells can be stored at -20 °C or 4 °C until use.

## Buffer recipes for sample preparation

| **4% formaldehyde in PBS** | For 10 mL of solution |
|---|---|
| 4% formaldehyde | 2.5 mL of 16% formaldehyde |
| 1$\times$ PBS | 1 mL of 10$\times$ PBS |
| | Fill up to 10 mL with molecular biology grade $H_2O$ |

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. Aspirate EtOH and air dry samples at room temperature.
2. Wash samples two times with 300 $\mu$L of 2$\times$ SSC.
3. Pre-hybridize samples in 300 $\mu$L of 30% probe hybridization buffer for 30 min at 37 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
4. Prepare probe solution by adding 1.2 pmol of each probe mixture (e.g. 1.2 $\mu$L of 1 $\mu$M stock) to 300 $\mu$L of 30% probe hybridization buffer at 37 °C.
   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.*
5. Remove the pre-hybridization solution and add the probe solution.
6. Incubate samples overnight (12–16 h) at 37 °C.
7. Remove excess probes by washing 4 $\times$ 5 min with 300 $\mu$L of 30% probe wash buffer at 37 °C.
   CAUTION: *probe wash buffer contains formamide, a hazardous material.*
   NOTE: *Wash solutions should be pre-heated to 37 °C before use.*
8. Wash samples 2 $\times$ 5 min with 5$\times$ SSCT at room temperature.

### Amplification stage

1. Pre-amplify samples in 300 $\mu$L of amplification buffer for 30 min at room temperature.
2. Separately prepare 18 pmol of hairpin H1 and 18 pmol of hairpin H2 by snap cooling 6 $\mu$L of 3 $\mu$M 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.*
3. Prepare hairpin solution by adding all snap-cooled hairpins to 300 $\mu$L of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate samples overnight (12–16 h) in the dark at room temperature.
   NOTE: *For dHCR imaging, amplify for 45-min to ensure single-molecule dots are diffraction-limited.*
6. Remove excess hairpins by washing 5 $\times$ 5 min with 300 $\mu$L of 5$\times$ SSCT at room temperature.
7. Aspirate 5$\times$ SSCT and add $\approx$100 $\mu$L of SlowFade Gold antifade mountant with DAPI.
8. Samples can be stored at 4 °C protected from light prior to imaging.

## 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 $\mu$L of 10% Tween 20 |
| | Fill up to 40 mL with ultrapure $H_2O$ |

## Reagents and supplies

Molecular biology grade $H_2O$ (Corning Cat. # 46-000-CV)
16% Formaldehyde (w/v), Methanol-free (Life Technologies Cat. # 28906)
DPBS, no calcium, no magnesium (Life Technologies Cat. # 14190144)
10× PBS (Ambion Cat. # AM9624)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
50% Tween 20 (Life Technologies Cat. # 00-3005)
ibidi $\mu$-slide ibitreat (ibidi Cat. # 80826)
SlowFade Gold antifade mountant with DAPI (Life Technologies Cat. # S36938)

## 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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