Molecular Technologies
MOUSE EMBRYOS

In situ HCR v2.0 protocol for whole-mount mouse embryos (*Mus musculus*)

This protocol has been optimized for embryos at stage E9.5.
Technical support: support@moleculartechnologies.org

Sample preparation

1. Wipe all dissection equipment with RNaseZap.
2. Kill a pregnant female mouse using an IACUC-approved protocol.
3. Immediately remove the uterus and submerge it in 4% paraformaldehyde (PFA) in a fresh RNase-free petri dish.
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
   NOTE: *Use fresh PFA and cool to 4 °C before use to avoid increased autofluorescence.*
4. Dissect the mouse embryos from the uterus while it is submerged in 4% PFA.
   CAUTION: *Perform this step with active ventilation from a fume hood or a histology slot hood to prevent exposure to the fixative fumes.*
   NOTE: *Each female mouse produces 6–9 embryos. For Steps 5–9, we recommend using 1 mL of solution per group of 10 embryos.*
5. Transfer the embryos to a clean vial containing 1 mL of fresh 4% PFA and fix them overnight or longer at 4 °C.
6. Wash 2 × 5 min with 1 mL of PBST on ice.
7. Dehydrate embryos into methanol (MeOH) with a series of graded 1 mL MeOH / PBST washes for 10 min on ice:
   (a) 25% MeOH / 75% PBST
   (b) 50% MeOH / 50% PBST
   (c) 75% MeOH / 25% PBST
   (d) 100% MeOH
   (e) 100% MeOH.
8. Store embryos at -20 °C overnight (> 16 h) or until use.
   NOTE: *Embryos could be stored for six months at -20 °C .*

Buffer recipes for sample preparation

4% Paraformaldehyde (PFA)	For 25 mL of solution
4% PFA	1 g of PFA powder
1× PBS	25 mL of 1× PBS
	Heat solution at 50–60 °C to dissolve powder

PBST	For 50 mL of solution
1× PBS	5 mL of 10× PBS
0.1% Tween 20	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 embryos.*

Multiplexed in situ HCR v2.0 protocol

Detection stage

1. Transfer the desired number of embryos for an experiment to a 1.5 mL eppendorf tube.
   NOTE: *All solutions and washes use a volume of 1 mL unless specified.*
2. Rehydrate with a series of graded MeOH/PBST washes for 10 min each at room temperature:
   (a) 75% MeOH / 25% PBST
   (b) 50% MeOH / 50% PBST
   (c) 25% MeOH / 75% PBST
   (d) 100% PBST
   (e) 100% PBST.
3. Immerse embryos in 10 μg/mL proteinase K solution for 15 min at room temperature.
   NOTE: *Proteinase K concentration and treatment time should be reoptimized for each batch of proteinase K, or for samples at a different developmental stage.*
4. Wash embryos 2 $\times$ 5 min with PBST.
5. Postfix with 4% PFA for 20 min at room temperature.
   CAUTION: *Use PFA with extreme care as it is a hazardous material.*
6. Wash embryos 3 $\times$ 5 min with PBST.
7. For each sample, transfer 1–4 embryos to a 1.5 mL eppendorf tube.
8. Incubate embryos in 1 mL solution containing 50% of hybridization buffer (with tRNA) and 50% of PBST until embryos are settled.
9. Pre-hybridize with probe hybridization buffer (with tRNA) for 30 min at 45 °C.
   CAUTION: *probe hybridization buffer contains formamide, a hazardous material.*
10. Prepare probe solution by adding 2 pmol of each probe (2 μL of 1 μM stock per probe) to 500 μL of probe hybridization buffer at 45 °C.
11. Remove the pre-hybridization solution and add the probe solution.
12. Incubate embryos overnight (12–16 h) at 45 °C.
13. Remove excess probes by washing with 500 μL of probe wash buffer at 45 °C:
    (a) 2 $\times$ 5 min
    (b) 2 $\times$ 30 min

    CAUTION: *probe wash buffer contains formamide, a hazardous material.*
    NOTE: *Wash solutions should be pre-heated to 45 °C before use.*
14. Wash with 500 μL of 50% probe wash buffer / 50% 5$\times$ SSCT for 10 min at 45 °C.
15. Wash embryos 3 $\times$ 5 min with 5$\times$ SSCT at room temperature.

Amplification stage

1. Pre-amplify embryos with 1 mL of amplification buffer for 30 min at room temperature.
2. Separately prepare 30 pmol of hairpin H1 and 30 pmol of hairpin H2 by snap cooling 10 μL of 3 μM stock in hairpin storage buffer (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 500 μL of amplification buffer at room temperature.
4. Remove the pre-amplification solution and add the hairpin solution.
5. Incubate the embryos overnight (12–16 h) in the dark at room temperature.
6. Remove excess hairpins by washing with 500 μL of 5× SSCT at room temperature:
   (a) 2 × 5 min
   (b) 2 × 30 min
   (c) 1 × 5 min

Sample clearing and mounting for microscopy

1. Clear embryos for imaging by incubating them in 500 μL of Scale A2 solution for 1 d in the dark at 4 °C.
2. Clean a 25 mm × 75 mm glass slide and a 22 mm × 30 mm No. 1 coverslip with RNase*Zap*. Add four beads of vaseline to each slide to support a coverslip at the corners.
3. Place and orient an embryo on a slide.
4. Remove excess buffer and add ≈100 μL of SlowFade Gold antifade mountant over the embryo.
5. Place the coverslip over the embryo. Apply enough pressure to push the coverslip onto the embryo without flattening it. Seal the edges of the coverslip with nail polish.
6. Store the slides in the dark at 4 °C until imaging.
7. A Zeiss 710 NLO inverted confocal microscope equipped with an LD LCI Plan-Apochromat 25×/0.8 Imm Corr DIC objective was used in our lab to acquire mouse images.

Buffer recipes for in situ HCR v2.0

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.

Proteinase K solution	For 2 mL of solution
10 μg/mL proteinase K	1 μL of 20 mg/mL proteinase K Fill up to 2 mL with PBST
Probe hybridization buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin
1× Denhardt's solution	800 μL of 50× Denhardt's solution
10% dextran sulfate	8 mL of 50% dextran sulfate
500 μg/mL tRNA	200 μL of 100 mg/mL tRNA Fill up to 40 mL with ultrapure H_2O
Probe wash buffer	For 40 mL of solution
50% formamide	20 mL formamide
5× sodium chloride sodium citrate (SSC)	10 mL of 20× SSC
9 mM citric acid (pH 6.0)	360 μL 1 M citric acid, pH 6.0
0.1% Tween 20	400 μL of 10% Tween 20
50 μg/mL heparin	200 μL of 10 mg/mL heparin Fill up to 40 mL with ultrapure H_2O
Amplification buffer	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
10% dextran sulfate	8 mL of 50% dextran sulfate Fill up to 40 mL with ultrapure H_2O
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
50% dextran sulfate	For 40 mL of solution
50% dextran sulfate	20 g of dextran sulfate powder Fill up to 40 mL with ultrapure H_2O
Scale A2	For 40 mL of solution
4 M urea	12.012 g of urea
10% glycerol	5 mL of glycerol
0.1% Triton X-100	50 μL of Triton X-100 Fill up to 50 mL with ultrapure H_2O Adjust pH to 7.7 with NaOH

Reagents and supplies

RNaseZap (Ambion Cat. # AM9780)
Paraformaldehyde (PFA) (Sigma Cat. # P6148)
Methanol (Mallinckrodt Chemicals Cat. # 3016-16)
Proteinase K, molecular biology grade (NEB Cat. # P8107S)
Formamide (Deionized) (Ambion Cat. # AM9342)
20× sodium chloride sodium citrate (SSC) (Life Technologies Cat. # 15557-044)
Heparin (Sigma Cat. # H3393)
50% Tween 20 (Life Technologies Cat. # 00-3005)
50× Denhardt's solution (Life Technologies Cat. # 750018)
Dextran sulfate, mol. wt. $>$ 500,000 (Sigma Cat. # D6001)
tRNA from baker's yeast (Roche Cat. # 10109509001)
UltraPure urea (Life Technologies Cat. # 15505-035)
Glycerol, ACS grade (Mallinckrodt Cat. # 5092-16)
Triton X-100 (Sigma Cat. # X100)
25 mm × 75 mm glass slide (VWR Cat. # 48300-025)
22 mm × 30 mm No. 1 coverslip (VWR Cat. # 48393-026)
SlowFade Gold antifade mountant (Life Technologies Cat. # S36936)

Citation Notes

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- **In situ HCR 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; qHCR flow cytometry – analog mRNA relative quantitation for high-throughput single-cell analysis; dHCR imaging – digital mRNA absolute quantitation. Protocols for v3.0 in diverse organisms are adapted from the zoo paper.

- **Quantitative HCR (qHCR) imaging**
  mRNA relative quantitation with subcellular resolution in the anatomical context of whole-mount vertebrate embryos; read-out/read-in analysis framework (Trivedi *et al.*, 2018).

- **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

- **Single-molecule HCR imaging**
  Single-molecule mRNA imaging 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).

- **In situ HCR v2.0**
  2nd generation in situ HCR 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).

- **Shielded covalent (SC) probes**
  Highly selective covalent capture of DNA and RNA targets, overcoming the longstanding tradeoff between selectivity and durable target capture (Vieregg *et al.*, 2013).

- **In situ HCR v1.0**
  1st generation in situ HCR 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).

- **Hybridization chain reaction (HCR) mechanism**
  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. *bioRxiv*, doi: http://dx.doi.org/10.1101/285213. (pdf, supp info)

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

Schwarzkopf, M., & Pierce, N. A. (2016). Multiplexed miRNA northern blots via hybridization chain reaction. *Nucleic Acids Res*, **44**(15), e129. (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**, doi:10.1242/dev.156869. (pdf, supp info, Read-out/Read-in 1.0 package)

Vieregg, J. R., Nelson, H. M., Stoltz, B. M., & Pierce, N. A. (2013). Selective nucleic acid capture with shielded covalent probes. *J Am Chem Soc*, **135**(26), 9691–9699. (pdf, supp info)