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HCR-FISH on mouse brain sections

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We use this protocol and it's working



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Abstract

This protocol provides a starting point for localization and visualization of RNAs in mouse brain tissue, using hybridization chain reaction (HCR)-amplified RNA-FISH (HCR-FISH). This can be useful for marking cell types of interest or investigating the effect of experimental manipulations on RNA expression.

This protocol is compatible with multiple downstream analysis processes, including microscopy and laser capture microdissection, and can be used with free-floating tissue or tissue that has already been mounted onto a slide (but be sure to choose the option that is best suited for your downstream application). This protocol can also be combined with immunohistochemical (IHC) detection of proteins in mouse brain tissue (e.g. <https://www.protocols.io/view/immunohistochemistry-ihc-on-mouse-brain-slices-5qpvokmq7l4o/v1>). If combining HCR-FISH and IHC, brief fixation of slices in 4% PFA after HCR-FISH may help in preserving HCR-FISH signal.



Materials

- ⊗ UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977023**
- ⊗ PBS - Phosphate-Buffered Saline (10X) pH 7.4 **Thermo Fisher Scientific Catalog #AM9625**
- ⊗ Triton X-100, 10% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #93443**
- ⊗ Tween 20, 10% aqueous solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #11332465001**
- ⊗ SSC (20X), RNase-free **Thermo Fisher Catalog #AM9763**
- ⊗ Ethylene carbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E26258**
- ⊗ Dextran Sulfate, 50% solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #3730-OP**
- ⊗ ProLong Diamond Antifade Mountant **Thermo Fisher Scientific Catalog #P36970**
- ⊗ Diethyl pyrocarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D5758**
- ⊗ Dimethyl dicarbonate (dimethyl pyrocarbonate) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #8187580100**
- ⊗ Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water **Thermo Fisher Scientific Catalog #H3570**
- ⊗ DAPI **Thermo Fisher Scientific Catalog #62248**

HCR FISH probes - can be obtained as ssDNA oligos, from Integrated DNA Technologies (IDT; <https://www.idtdna.com/>) and/or from Molecular Instruments (<https://www.molecularinstruments.com/>)

HCR hairpins - can be obtained from Molecular Instruments (<https://www.molecularinstruments.com/>)



Before start

This protocol assumes that you are starting from fixed free-floating brain slices kept in RNase-free 1x PBS at  4 °C , or from fixed sections mounted on a glass slide, kept at  -70 °C or below. Free-floating sections may also be kept at  -20 °C in 100% ethanol. In this case, gradually rehydrate the tissue in 70% then 50% ethanol, and wash twice with RNase-free 1x PBS for  00:15:00 each before starting the protocol.

We typically process free-floating sections  30 µm to  100 µm thick, and mounted sections  5 µm to  100 µm thick.

Free-floating sections may allow for better penetration of reagents into thick sections (as diffusion occurs from both sides). Mounting sections onto a slide may be more convenient for some sectioning methods (cryosectioning) and can enable thinner sections to be collected without risk of damaging sections during processing. Depending on the size of well-plate used, mounted sections may use less antibody, and may thus be cheaper to process. Mounting sections on slides is also recommended if incorporating an antigen retrieval step (e.g. if combining IHC and HCR-FISH).



Reagent set up

1 Note on RNA integrity

Note

In order to preserve RNA integrity in tissue, use RNase-free reagents and/or to treat reagents with an RNase-inhibitor (DEPC or DMPC). Similarly, use RNase-free consumables and filtered pipette tips.

Ideally, tissue should be kept in RNase-free buffers starting from the post-fixation washes and slicing.

Dimethyl pyrocarbonate (DMPC) is a less toxic alternative to diethyl pyrocarbonate (DEPC) that can be used in the same manner. To treat water or buffers with DMPC (or DEPC), add 1/1000 DMPC to a glass bottle with the buffer (i.e.  1 mL of DMPC to  1000 mL of water). Shake to mix with lid tightly closed. Loosen cap and let sit  Overnight in a fume hood (or if needed sooner,  02:00:00 at  37 °C). Autoclave to inactivate the DMPC and allow to cool to room temperature before using.

Note: DMPC and DEPC cannot be used to treat buffers containing Tris (this protocol does not use any Tris-buffered solutions).

2 Prepare RNase-free 1xPBS by combining:

- RNase-free 10x PBS:  50 mL
- UltraPure water:  450 mL

Invert to mix. Store at  Room temperature for up to 3 months.

Note

Scale up or down volumes based on needs.

3 Prepare RNase-free 1xPBS with 0.1% Triton X-100 (PBSTx), by combining:

- RNase-free 10x PBS:  5 mL



- 10 % Triton X-100: 500 μ L
- UltraPure water: 44.5 mL

in a 50 mL conical tube. Invert to mix. Store at Room temperature for up to 3 months.

Note

Scale up or down volumes based on needs.

4 Prepare probe hybridization buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 5 mL
- 50% dextran sulfate: 10 mL
- UltraPure water: 30 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 weeks, or -20 °C for up to a year.

Note

Scale up or down volumes based on needs. We make appropriately sized aliquots (e.g. 10 mL) and store at -20 °C .

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based hybridization buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

Ethylene carbonate is solid at room temperature. Pre-warm to 37 °C to melt.

Probe hybridization buffer can also be obtained from Molecular Instruments (<https://www.molecularinstruments.com/>)

5 Prepare stringent wash buffer, by combining:



- 20x saline-sodium citrate (SSC) buffer: 5 mL
- Ethylene carbonate: 15 mL
- UltraPure water: 30 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 weeks, or -20 °C for up to a year.

Note

Scale up or down volumes based on needs. We make appropriately sized aliquots (e.g. 10 mL) and store at -20 °C .

Ethylene carbonate is a non-toxic substitute for formamide, and may also help to reduce background from non-specific interaction (as compared to formamide-based wash buffers). Substituting ethylene carbonate for formamide, at the same concentration, should yield qualitatively similar results.

Stringent wash buffer can also be obtained from Molecular Instruments (as "Probe wash buffer"; <https://www.molecularinstruments.com/>)

6 Prepare wash buffer (5x SSC, with 0.1% Tween-20), by combining:

- 20x saline-sodium citrate (SSC) buffer: 125 mL
- 10% Tween-20: 5 mL
- UltraPure water: 370 mL

Store solution at Room temperature for up to a year.

7 Prepare HCR amplification buffer, by combining:

- 20x saline-sodium citrate (SSC) buffer: 5 mL
- 50% dextran sulfate: 10 mL
- UltraPure water: 35 mL

in a 50 mL conical tube. Store solution at Room temperature for up to 3 months.

**Note**

Scale up or down volumes based on needs.

HCR amplification buffer can also be obtained from Molecular Instruments (<https://www.molecularinstruments.com/>)

- 8 This protocol diverges depending on whether you are processing free-floating sections or mounted sections.

Once samples have some fluorescence, keep samples in the dark. E.g. if samples have endogenous fluorescence (e.g. GFP or tdTomato-expression), keep samples in dark through the entire protocol. If fluorescence is introduced with fluorophore-conjugated hairpins, keep samples in dark during and after the amplification step.

STEP CASE**Free-floating sections**

20 steps

- 9 Note on scaling and volumes

Note

The volumes in this protocol are appropriate for a 24-well plate with 12 or fewer sections per well. If processing more sections per well, consider using a larger well size (e.g. a 12-well plate), and scale up volumes appropriately. If processing fewer sections per well, consider using a smaller well size (e.g. a 48-well plate) and scale down volumes appropriately.

Appropriate scaling factors can be found in <https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cell-culture-useful-numbers.html>, based on ratio between surface areas.

For free-floating sections, incubations and washes can be done in the wells.

- 10 Note on serial sections

**Note**

In some cases, it may be desirable to arrange sections on a slide serially. If processing free-floating sections, it can be helpful to separate sections across multiple wells during sectioning (e.g. Starting at well A1, collect section 1. Then collect section 2 in well A2. Section 3 in A3, Section 4 in A4, etc. When collecting section 7, start back at the beginning and place in A1. Section 8 in A2, etc.)

This ensures that serial sections in the same well are separated by some distance and can be easily placed on the slide in the correct order.

If left and right side are important, mark one side. E.g. trim tissue off in a region outside of the region of interest.

- 11 Before beginning, pre-warm hybridization buffer to 37 °C . For each well of a 24-well plate, you will need 1 mL of hybridization buffer. Pre-warm extra (~20%) to ensure that there is enough for each well.
- 12 If necessary, transfer the desired slices to a new well plate filled with RNase-free 1x PBS. Use a paintbrush to transfer sections.
- 13 Aspirate and dispose of 1x PBS in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

1h

Add 500 µL of PBSTx to each well. Incubate for 01:00:00 at Room temperature with gentle shaking.

Note

This step helps to permeabilize tissue, enabling penetration of staining reagents into tissue. Depending on the tissue and treatment (e.g. if the tissue was kept in ethanol), this step may not be necessary.

- 14 Equilibrate tissue in probe hybridization buffer.

Aspirate and dispose of 1x PBSTx in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add 500 µL of pre-warmed probe hybridization buffer to each well, and incubate at 37 °C for 01:00:00 .

- 15 In the last 5 minutes of the probe hybridization buffer equilibration step, prepare the probe solution.

Dilute DNA probes against target RNA of interest in prewarmed hybridization buffer, to a final concentration of **1M** 2 nanomolar (nM) . Vortex to mix and keep at **37 °C** until use.

Note

For thicker tissue sections, a higher probe concentration (e.g. 4 nM) may be desirable.

Probe sets targeting multiple RNAs of interest can be combined at this step, assuming that they have separate HCR initiator sequences and can be detected orthogonally.

- 16 Following equilibration in probe hybridization, add probe solution.

Aspirate and dispose of probe hybridization buffer in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add **500 µL** of probe solution (i.e. probe hybridization buffer + probes) to each well. Gently tap the edge of the plate to ensure tissue is surrounded by probe solution. Use parafilm to seal the edge of the plate and prevent evaporation. Incubate **Overnight** at **37 °C** .

Note

During this step, the DNA probes hybridize to the target RNA through complimentary base-pairing.

- 17 Following overnight probe hybridization, pre-warm stringent wash buffer to **37 °C** . For each well of a 24-well plate, you will need **3 mL** of stringent wash buffer. Pre-warm extra (~10%) to ensure that there is enough for each well. Mix or vortex a couple times during the pre-warming step to ensure buffer is thoroughly mixed.

- 18 Aspirate and dispose of probe solution in wells. For wells being stained with the same probe set (or combination of probe sets), use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

1h



Quickly rinse each well with pre-warmed stringent wash buffer, by adding  1 mL of stringent wash buffer to each well, gently tapping the edge of the plate, then aspirating and disposing of that wash buffer.

Following this rinse, add  1 mL of pre-warmed stringent wash buffer to each well.

Incubate for  00:30:00 at  37 °C .

Repeat this  00:30:00 wash step 1 more time.

- 19 After stringent washes, perform 2 washes with wash buffer (5x SSC, with 0.1% Tween-20).

30m

Aspirate and dispose of stringent wash buffer in each well. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Quickly rinse each well with wash buffer, by adding  1 mL of wash buffer to each well, then aspirating and disposing of that wash buffer.

Wash samples 2 times, with  1 mL of wash buffer per wash. Each wash should be

 00:30:00 at  Room temperature , with gentle shaking.

- 20 Equilibrate tissue in amplification buffer.

Aspirate and dispose of wash buffer in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add  500 µL of amplification buffer to each well, and incubate at

 Room temperature for  01:00:00 .

- 21 During HCR amplification buffer equilibration step, prepare HCR hairpins.

12m

1. Aliquot each hairpin separately into PCR tubes

2. Using a thermocycler, heat hairpins to  95 °C for  00:02:00

3. Remove hairpins from thermocycler and allow to cool back down to room temperature in the dark (at least  00:10:00

Note

Snap-cooling hairpins is critical, as it allows the hairpins to re-fold properly.

CRITICAL Do not mix hairpins together before snap-cooling, as this may cause them to hybridize to one another. Only mix the hairpins together in amplification buffer, and only once they have cooled down to room temperature.

- 22 Following equilibration in HCR amplification buffer and snap-cooling hairpins, prepare HCR amplification solution. For each well, prepare  500 μL of amplification solution (plus 10% extra). Dilute snap-cooled hairpins to  60 nanomolar (nM) in the HCR amplification buffer.

For example, for one well with one probe set using the B5 initiator, combine:

- HCR amplification buffer:  480 μL
- Hairpin 1 (e.g. B5H1 @  3 micromolar (μM)):  10 μL
- Hairpin 2 (e.g. B5H2 @  3 micromolar (μM)):  10 μL

Briefly vortex to mix.

- 23 Aspirate and dispose of amplification buffer in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add  500 μL of amplification solution (i.e. amplification buffer plus hairpins) to each well. Gently tap the edge of the plate to ensure tissue is surrounded by probe solution. Seal edge of plate with parafilm and incubate  Overnight at  Room temperature in dark, with gentle shaking.

- 24 Following HCR amplification, aspirate and dispose of amplification solution in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Quickly rinse each well with wash buffer (i.e. 5x SSC, with 0.1% Tween-20), by adding  1 mL of wash buffer to each well, gently tapping the edge of the plate, then aspirating and disposing of that wash buffer.

Wash samples 2 times, with  1 mL of wash buffer per wash. Each wash should be  00:30:00 at  Room temperature , with gentle shaking.



- 25 Following washes in 5x SSC, with 0.1% Tween-20, aspirate and dispose of wash buffer in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Quickly rinse each well with 1x PBS, by adding  1 mL of 1x PBS to each well, gently tapping the edge of the plate, then aspirating and disposing of that wash buffer.

Add  1 mL of 1x PBS to each well and store tissue at  4 °C in the dark until proceeding with mounting or additional staining.

- 26 (Optional) Label proteins in sample using IHC (e.g. <https://www.protocols.io/view/immunohistochemistry-ihc-on-mouse-brain-slices-5qpvmq7l4o/v1>).

If combining HCR-FISH and IHC, it may be beneficial to briefly fix sections with 4% PFA to preserve localization of labeled HCR hairpins.

- 27 (Optional) Stain sections with Hoechst 33342 or DAPI to label nuclei. For each well, prepare  500 µL of 1x PBS with 1/10000 Hoechst 33342 or DAPI.

20m

Aspirate and dispose of 1x PBS in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Add  500 µL of Hoechst or DAPI solution. Incubate  00:15:00 at  Room temperature with gentle shaking.

Following incubation with Hoechst or DAPI solution, aspirate and dispose of Hoechst or DAPI solution in wells. Use the same pipette tip for each well, but be careful not to transfer tissue between wells. Ensure complete removal.

Wash samples 3 times, with  1 mL of 1x PBS per wash. Each wash should be  00:05:00 at  Room temperature, with gentle shaking.

- 28 Mount sections on slide. Use paintbrush to transfer and flatten sections onto surface of slide. Once all sections are placed, excess 1x PBS can be removed with a Kimwipe and/or by placing slide in a fume hood. Allow sections to dry on slide (sections are dried once the tissue appears transparent).

Once tissue is dry, pipette  100 µL of mounting media in a line on the surface of the slide, near the tissue sections. Spread the mounting media over the tissue by gently lowering a clean coverslip over the tissue, being careful not to introduce bubbles.



Once all coverslips are mounted, gently press down on each coverslip to push out excess mounting media. Store in the dark at  Room temperature for at least  Overnight until mounting media cures. Once cured, excess Prolong Diamond can be easily removed by running slides under a gentle stream of MilliQ water, being careful to not displace coverslips.

Note

Be careful not to dry tissue too long. If the edges of the tissue are turning white, the tissue is becoming too dry.

For mounting coverslips, we use Prolong Diamond, but any mounting medium compatible with fluorescence signal should work. If using another mounting medium, follow manufacturer directions for that mounting medium.