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RNA In situ hybridization (ISH) using RNAscope®

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We use this protocol and it's working

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Abstract

This protocol outlines the procedures for RNA in situ hybridization to simultaneously detect up to three targets using the RNAscope® Multiplex Fluorescent v2 assay (ACD). The steps adhere to the manufacturer's guidelines (Doc. No: 323100-USM) with custom modifications optimized for fixed frozen mouse brain sections.

Attachments

DOCX

Ding_Lab_RNA In situ...

23KB

Materials

o RNAscope Target Probes: C1, C2, C3 probes
o Probe dilute (Cat. No. 300041)
o RNAscope Multiplex Fluorescent Reagent Kit v2 (Cat. No. 323270)
o TSA plus fluorophores: Fluorescein (NEL741001KT), Cyanine 3 (NEL744001KT), Cyanine 5 (NEL745001KT)
o 1X Phosphate-buffered saline (PBS)
o 4% Paraformaldehyde (PFA): Freshly prepared in 1X PBS
o 30% Sucrose Solution: Prepared in 1X PBS.
o 5X SSC Buffer
o Optimal Cutting Temperature (OCT) Embedding Medium
o Ethanol: 50%, 70%, and 100% solutions
o Superfrost Plus microscopic slides
o Coverslips
o Mounting Media: Fluorescence-compatible, anti-fade mounting media
o Steamer
o Hybridization incubator
o Humidity Tray
o ImmEdge Hydrophobic Barrier Pen

Safety warnings

Wear appropriate PPE as required by your institution.

Ethics statement

Prior ethics approval (e.g. IACUC) should be obtained before performing these experiments. Approval was obtained by the Stanford University IACUC before any procedures were performed.

Sample Preparation

1 Perfuse mice with freshly prepared 4% paraformaldehyde (PFA) in 1X PBS.

2 Dissect the brain and fix in 4% PFA at 4 °C overnight.

3 Immerse the tissue in 30% sucrose in 1X PBS at 4 °C until the tissue sinks to the bottom of the container (approximately 48 HRS for brain tissue).

4 Freeze tissue in OCT embedding media with dry ice or liquid nitrogen, and store it in an airtight container at -80 °C.

5 Section tissue into 20 µm thick slices and mount on Superfrost Plus slides. Store at -80 °C or proceed to pretreatment.

Pretreatment (Day 1)

6 Wash slide in 1xPBS for 5 min

7 Bake slides at 60 °C for 30 min in a hybridization incubator.

8 Post-fix slides in 4% PFA in PBS for 15 min at 4 °C.

9 Dehydrate tissue by immersing slides sequentially in 50%, 70%, and 100% ethanol for 5 minutes each.
Note: Handle PFA in a chemical hood and collect waste from step 8-9 as biohazard.

10 Air dry slide for 5 min at Room temperature (RT).

11 Cover sections with RNAscope® Hydrogen Peroxide and incubate for 10 minutes at Room temperature.

12 Wash slides twice for 5 minutes each in 1X PBS

13 Preheat 1xPBS and RNAscope® 1X Target Retrieval Reagent in a steamer (> 99 °C).

14 Briefly immerse slides in PBS for 10 seconds, then transfer to the Target Retrieval Reagent. Cover the steamer immediately.

14.1 Steam slides for 3 mins.

15 Transfer slides to fresh PBS and rinse for 15 seconds.

16 Immerse slides in 100% ethanol for 3 minutes.

17 Air dry slides at Room temperature.

18 Draw barriers around sections with ImmEdge pen. Allow to dry completely.

19 Place slides in a humidity tray and cover sections with Protease III
Note: From this step forward, keep slides in the humidity tray for all incubations.

20 Incubate slides at 40 °C in a hybridization incubator for exactly 20 mins.

21 Wash slides **twice** for 5 minutes each in PBS.
Note: From this step forward, ensure sections do not dry out.

Probe Hybridization (Day 1)

22 Dilute and mix probes of target RNA (C1, C2 and/or C3 probes) using probe dilute (1:50~2000).
Note: Do not mix probes from the same channel.

23 Apply probe mixture on slides to cover all sections.

24 Incubate at 40 °C for 2 hours.

25 Wash slides twice for 2 minutes each in 1x RNAscope® Wash Buffer.

26 Store slides in 5x SSC buffer overnight at Room temperature.

Signal Amplification (Day 2)

27 Hybridize AMP1: Apply AMP1 to cover sections. Incubate at 40 °C for 30 minutes. Wash twice for 2 minutes each in Wash Buffer.

28 Hybridize AMP2: Apply AMP2 to cover sections. Incubate at 40 °C for 30 minutes. Wash twice for 2 minutes each in Wash Buffer.

29 Hybridize AMP3: Apply AMP3 to cover sections. Incubate at 40 °C for 15 minutes. Wash twice for 2 minutes each in Wash Buffer.

Fluorescence Signal Development (Day 2)

30 **Note:** Fluorescence signals are developed sequentially for each channel. The steps below use HRP-C1 as an example. Select the appropriate HRP channel for your probes. Channels and fluorophores (Fluorescein, Cy3 and Cy5) can be paired as desired.

31 Apply HRP-C1 to cover sections. Incubate at 40 °C for 15 minutes. Wash twice for 2 minutes each in Wash Buffer.

32 Dilute TSA fluorophores at 1:1000 (Fluorescein, Cy3 or Cy5) in TSA buffer.

33 Apply diluted fluorophore to cover sections. Incubate at 40 °C for 30 minutes. Wash twice for 2 minutes each in Wash Buffer.

34 Apply HRP Blocker to cover sections. Incubate at 40 °C for 15 minutes. Wash twice for 2 minutes each in Wash Buffer.

35 Repeat steps 31–34 for C2 and/or C3 probes with the appropriate HRP and fluorophores.

Counterstaining and mounting (Day 2)

36 Apply DAPI to cover sections and incubate for 30 seconds at Room temperature.

37 Remove DAPI by tapping or flicking the slides, and immediately add 1–2 drops of mounting media on each slide.

38 Place coverslips over the tissue, avoiding air bubbles.

39 Dry slides in dark and store at 4 °C.