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Fresh Frozen RNAScope Protocol

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We use this protocol and it's working

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Abstract

A refined protocol for ACD Bio RNAscope Multiplex Fluorescent v2 in situ hybridization (ISH), tailored for reliable use with fresh-frozen mouse brain tissue. This guide provides a step-by-step workflow, including essential sample preparation and cryosectioning methods. The protocol is validated specifically for the mouse brain, but may be applied to other central nervous system areas.

Guidelines

1. Under-fixation of tissue specimens will result in protease over-digestion, which leads to loss of RNA and poor tissue morphology. Over-fixed tissue specimen will result in protease under-digestion, which leads to poor probe accessibility and low signal and signal/background ratio while maintaining excellent tissue morphology.

2. Fixed frozen at 7-15um sections, tissue pretreatment using Target Retrieval and Protease III

3. Depends on the probe, but I’ve noticed signal fades away 2 weeks after perfusion. Try to do all steps as soon as possible after perfusion.

Materials

Materials
- 1x PBS (using autoclaved H2O)
- DDW
- OCT Compound or FSC 22 Clear (Leica, #3801480)
- 2-methylbutane
- methanol
- SuperFrost Plus slides (Fisherbrand, #1255015)
- Microscope Cover Glass 24×60mm No. 1.5 (Fisherbrand, #12541037)
- 4% PFA in 1x PBS
- Fresh 50%, 70%, 100% EtOH
- ImmEdge Hydrophobic Barrier Pen
- Target Probes in C1, C2 and/or C3
- Positive and/or Negative Control Probes
- Probe Diluent (Cat #300041) if using C2 or C3 without C1
- RNAScope™ Multiplex Fluorescent Reagent Kit v2 (ACD Bio# 323100)
  - RNAScope Hydrogen Peroxide
  - Protease Plus
  - 50x Wash Buffer
  - RNAScope Multiplex FL v2 AMP 1,2,3
  - HRP-C1, HRP-C2, HRP-C3, HRP blocker
  - TSA Buffer
  - DAPI
- Akoya Biosciences Opal fluorophores (Opal 520 #FP1487001KT, Opal 570 #FP1488001KT, Opal 690 #FP1497001KT)
- HybEZ Hybridization System
- ProLong Gold Antifade Reagent from Life Technologies (P36930)
- RNase away and 70% EtOH for cleaning surfaces during section preparation (everything you use should be cleaned. Be careful of RNA degradation prior to fixation w PFA

Section Preparation

1 Perfuse mouse with 1xPBS and harvest brain (for younger mice extract brain without perfusing). ~7ml/min perfusion rate with pump. 1xPBS volume = weight (g) (ex. For 20g mouse, use 20ml of 1xPBS)

2 Immediately place brain into OCT mold, cover with OCT, and freeze in a metal beaker filled with 2-methylbutane, surrounded by dry ice in methanol.

3 Keep brain at -80C overnight. Section on the cryostat at 16 µm thickness onto SuperFrost Plus slides. Keep slides at -80C overnight.

Sample Fixation

4 Immerse slides in 4% PFA in 1xPBS, incubate slides for 2 hours (at least 15 min) at RT.

5 Wash x2 in 1xPBS

Dehydrate the Tissue

6 Immerse slides in 50% EtOH in DDW for 5 min at RT

7 Immerse slides in 70% EtOH in DDW for 5 min at RT

8 Immerse slides in 100% EtOH in DDW for 5 min at RT

9 Immerse slides in 100% EtOH in DDW for 5 min at RT

9.1 If necessary, store the slides in 100% EtOH at –20°C for up to 1 WEEK, but not recommended

10 Let slides dry and draw a barrier around sections at RT ~1 min

Make sure the hydrophobic barrier remains intact so that the tissues do not dry at any time.

11 *Turn on HybEZ oven at 40C with Humidity Control Tray inside (mode RNAScope) to prepare for hybridization step. Place wet paper inside the tray.*

Tissue Pretreatment

12 Add drops of RNAScope Hydrogen Peroxide. Incubate for 10 min at RT

13 Wash in DDW for 2 min x2

14 Add drops of Protease Plus. Incubate for 10 min at RT (100–150 µL is 4–6 drops)

14.1 While incubating, warm up your probes (warmed for 10 min at 40C, RT for 10 min) and 50x wash buffer. Precipitation occurs during storage.

15 Wash in 1xPBS for 2 min x2

Hybridization

16 Prepare probe master mix of C1:C2:C3 of 50:1:1. For ~4 sections per slide, ~100µL of C1, 2µL C2, 2µL C3. Include positive/negative control if possible.

17 Place slides locked in the Slide Holder into the Humidity Control Tray.

18 Add appropriate amount of probe mix to entirely cover each slide. Close tray and insert into oven for 2 hours at 40C.

19 While incubating:
1) Set a 1 hour 30 min timer to bring out the AMP boxes 30 min before the probe step is over, to bring the AMPs to RT.
2) Prepare fresh 1x wash buffer (490ml DI water and 10ml of 50x wash buffer)

20 Wash in 1x wash buffer for 2 min x2

21 *If necessary, STOPPING POINT – slides can be stored in 5x SSC buffer overnight at RT. Wash with 1x Wash buffer for 2 min at RT before continuing with assay, but not recommended*

Amplification

22 Place slides onto the slide holder.

23 Apply AMP1 and incubate in oven for 30 min at 40C

24 Wash in 1x wash buffer for 2 min x2

25 Apply AMP2 and incubate in oven for 30 min at 40C

26 Wash in 1x wash buffer for 2 min x2

27 Apply AMP3 and incubate in oven for 15 min at 40C

28 Wash in 1x wash buffer for 2 min x2

29 *During incubation: prepare 3 Opal secondaries – 1:750 in TSA buffer for Opal 520, 570, 690 and keep in dark. Equilibrate HRP-C1,-C2,-C3, and blockers to RT 30 min prior.*

Develop HRP Signal

30 Apply HRP-C1 and incubate in oven for 15 min at 40C

31 Wash in 1x wash buffer for 2 min x2

32 Apply diluted fluorophore for C1 and incubate in oven for 30 min at 40C

33 Wash in 1x wash buffer for 2 min x2

34 Apply block and incubate in oven for 15 min at 40C

35 Wash in 1x wash buffer for 2 min x2

36 Repeat Steps 30-35 for C2 and C3 if using more channels.

Counterstain and Mount Slides

37 Apply DAPI and incubate for 30s - 5 min at RT

38 Decant DAPI and immediately cover slides with Prolong Gold

39 Coverslip slides with a 1.5 thickness slide cover

40 Let slides dry overnight in a dark place at 4C. Scan when fully dry.