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Preparation and RNAscope-labeling of fresh mouse midbrain tissue

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ABSTRACT

Below we describe preparing and labeling coronal sections of mouse substantia nigra (SNc) and ventral tegmental area (VTA) for probes targeting the genes Slc17a6 (VGLUT2), Slc32a1 (VGAT), and Slc18a2 (VMAT2).

ATTACHMENTS

fresh frozen prep rnascope protocol.docx

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MATERIALS

Part A:

Material	Supplier and Catalog Number
RNAseZap	Invitrogen (AM9780)
Pentobarbital	Virbac
Scissors	Fine Science Tools (14058-09)
Forceps	Fine Science Tools (11152-10)
Culture tubes	Fisher Scientific (14-959-11)

Part B:

Material	Supplier and Catalog Number
Cryostat	Leica (CM3050S)
Anti-roll plate	Leica (14047742497)
Brushes	Electron Microscopy Sciences (66100-30)
OCT Medium	Sakura (4583)
Superfrost Plus Microscope Slides	Thermo Fisher Scientific (12-550-15)

Part C & D:

A	B
Material	**Supplier and Catalog Number**

	A	B
	Multiplex Florescent Kit	Advanced Cell Diagnostics (320851)
	Wash Buffer	Advanced Cell Diagnostics (310091)
	HybEZ Oven	Advanced Cell Diagnostics (310010)
	Pretreatment Kit (Protease)	Advanced Cell Diagnostics (310842)
	UltraPure DNase/RNase-Free Distilled Water	Invitrogen (10977049)
	Wash containers	Andwin Scientific (7154801)
	Safe Lock Centrifuge Tube, 1.5 mL	Eppendorf (0030123611)
	ImmEdge Hydrophobic Barrier PAP Pen	Vector Laboratories (H-4000)
	DAPI	Roche (10236276001)
	Fluoromount-G	Southern Biotech (0100-01)
	Coverslip	Corning (2980-225)
	Falcon 50 mL Conical Centrifuge Tubes	Fisher Scientific (352098)

A. Mouse brain extraction

1 Spray experimenter's gloves and all tools with RNAseZap before starting and in between brain extractions.

2 Add approximately 50mL of isopentane in a beaker (cleaned with RNAseZap) and chill on dry ice.

3 Anaesthetize mouse with pentobarbital (200 mg/kg i.p.).

4 Decapitate mouse and extract brain from skull.

5 If planning on collecting coronal sections, use a razor blade to block the brain caudal of your region of interest. For example, around -6 mm from bregma for midbrain sections.

6 Immediately snap freeze brain by submerging brain for 30 to 45 seconds in isopentane chilled on dry ice.

7 Place brains in labeled culture tubes pre-chilled on dry ice.

8 Frozen brains can be moved to either an RNAse-free cryostat at −18°C or stored at −80°C.

B. Sectioning

9 Set temperature of cryostat objective and chamber to −18°C.

10 Clean interior of cryostat with 100% ethanol.

11 Before sectioning, spray experimenter's gloves and tools with RNAseZap. Tools that directly touch sections (e.g. brushes, anti-roll plate) should be air dried completely before being placed in cryostat.

12 Place culture tube with brain in chamber and let it equilibrate to chamber temperature for about 10 minutes.

13 For coronal sections, mount caudal end of brain to chuck using OCT mounting medium. Mount brain as perpendicular to the horizontal plane of the chuck as possible. Equilibrate to chamber temperature again for about 10 minutes.

14 Place chuck with brain on objective and adjust orientation to make brain perpendicular to blade. As you begin cutting, adjust brain position so brain landmarks (e.g. corpus collosum and anterior commissure) are as symmetrical as possible. Ensure anti-roll plate is positioned correctly before reaching area of interest.

15 Cut coronal brain sections serially at 20 µm on a cryostat throughout the rostro-caudal extent of the region of interest (ROI). For example, SNc and VTA should be collected from -2.5 mm up to and including -4.1 mm from bregma using the Paxinos and Watson atlas (Paxinos & Franklin, 2001).

16 Directly mount sections onto Superfrost glass slides. Mount 4 sections per slide.

17 Air-dry at room temperature (RT) for 10-20 minutes, then store at -20°C for 2 hours.

18 Slides can be used for RNAscope assay or stored at −80°C in a slide box within an airtight container (e.g. Ziploc bag).

C. DAPI-containing Fluoromount-G preparation

19 Pipet DAPI to Fluoromount-G to reach a concentration of 0.5 µg/mL.

20 Pipet up and down several times to mix, then vortex for at least 30 seconds.

21 Wait at least 24 hours before use. Container should always be covered in foil and stored at 4°C.

D. RNAscope assay

22 Turn on HybEZ™ Oven (ACD) and set to 40°C.

23 Warm probes in heat-bath for 10 minutes at 40°C, then cool to RT.

24 Make probe mixture in autoclaved Eppendorf tube by pipetting (with autoclaved pipette tips) 50 parts C1 probe or diluent, 1 part C2 probe (optional) and 1 part C3 probe (optional). Prepare about 150 µL per slide, assuming each slide contains 4 coronal mouse sections.

25 Fix slides in RNAse-free PBS containing 4% paraformaldehyde at 4°C for 15 minutes.

26 Dehydrate slides in serial ethanol (EtOH) washes: 2 minutes in 50% EtOH, 2 minutes in 70% EtOH, 2 minutes in 100% EtOH twice. Make EtOH dilutions with ultrapure water in sterile conical tubes.

27 Air dry slides for 2 to 5 minutes.

28 Use hydrophobic pen to draw a barrier around sections. Air dry for 2 to 5 minutes.

29 Incubate slides with protease IV at RT for 30 min. Keep slides covered to prevent dust contamination.

30 Rinse slides in RNAse-free 1X PBS at RT.

31 Decant excess liquid from slides by gently tapping the slide edge on a paper towel. Pipette enough probe mixture to cover sections.

32 Incubate for 2 hours in the HybEZ™ Oven at 40°C.

33 Wash in 1X wash buffer for 2 minutes, twice.

34 Decant excess liquid from slides and add enough AMP1 to cover sections. Incubate for 30 minutes in the HybEZ™ Oven at 40°C.

35 Wash in 1X wash buffer for 2 minutes, twice.

36 Decant excess liquid from slides and add enough AMP2 to cover sections. Incubate for 15 minutes in the HybEZ™ Oven at 40°C.

37 Wash in 1X wash buffer for 2 minutes, twice.

38 Decant excess liquid from slides and add enough AMP3 to cover sections. Incubate for 30 minutes in the HybEZ™ Oven at 40°C.

39 Wash in 1X wash buffer for 2 minutes, twice.

40 Decant excess liquid from slides and add enough AMP4A to cover sections. Incubate for 15 minutes in the HybEZ™ Oven at 40°C.

41 Decant excess liquid from slides, add DAPI-containing Fluoromount-G, and coverslip.

42 Store slides in the dark at 4°C.