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RNAScope in situ hybridization (ISH) multiplex fluorescent protocol

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We use this protocol and it's working

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Abstract

Instructions to perform ISH using RNAscope kit from **a**cdbio company

Guidelines

ISH is used to detect RNA. Thus, it is very important that all the reagents, equipment, and bench space used are very clean and uncontaminated.

Materials

Reagents:

- RNAscope Fluorescent Multiplex Reagent Kit **ACD Catalog #**320850 - contains a **pretreatment kit** with RNAscope protease III RNAscope Protease III **ACD Catalog #**322337 and RNAscope protease IV ; a **detection kit** (AMP1, AMP2, AMP3, HPR-C1, HPR-C2, HPR-C3, HPR blockers + DAPI solution) and **wash buffer reagents** (50X wash buffer)

- RNAscope Target Retrieval (x10) **ACD Catalog #**322001

- Prolong Gold Antifade Mountant **Thermo Fisher Scientific Catalog #**P36930
- RNAscope probes kit (probes + RNAscope probe diluent)
- Distilled water

Equipment:

- cover glass 24x50mm
- tubes (various sizes)
- paper towel - absorbent paper
- foil
- hydrophobic pen, ImmEdge hydrophobic barrier pap pen **Vector Laboratories Catalog #**H-4000
- slice rack
- slice holder
- water bath
- incubator
- oven (see below)

Equipment	
HybEZ II Hybridization System (110 or 220V)	NAME
Oven	TYPE
ACDBio	BRAND
321711	SKU

- slice tray (see below)

Equipment	
HybEZ Humidity Control Tray	NAME
ACDBio	BRAND
310012	SKU

Solution

20x SSC buffer stock
- Dissolve 175.3 g of NaCl and 88.2 g of sodium Citrate in 800 ml of ddH2O
- Adjust pH to 7.0 with a few drops of 1M HCl (~ 20 drops)
- Adjust volume to 1L with additional ddH2O (pH becomes ~7.06)
- Sterilize by autoclaving
-5x SSC dilution can be stored for up to 2 months at RT

320293_RNAscope Multiplex_UM_0…

320535_TechNote_Fixed frozenFluor…

Protocol materials

RNAscope Protease III **ACD Catalog #**322337	Materials, Step 28
Prolong Gold Antifade Mountant **Thermo Fisher Scientific Catalog #**P36930	Materials
RNAscope Fluorescent Multiplex Reagent Kit **ACD Catalog #**320850	In Materials and 4 steps
RNAscope Target Retrieval (x10) **ACD Catalog #**322001	Materials, Step 14.1
ImmEdge hydrophobic barrier pap pen **Vector Laboratories Catalog #**H-4000	Materials, Step 27

Before start

For Fixed Frozen Brain samples

The challenge with thicker sections (30 to 40 microns) is tissue attachment. Hence, there are additional fixation and baking steps with respect to the standard protocol.

- Cut 30 µm (or 40 µm) sections.
- Collect sections into 12-24 well plates in anti-freeze solution
- Store sections at -20^{o}C for no more than 3-4 weeks; at 4°C for no more than 2 days.

For Fixed Frozen Nodose Ganglion samples

- Cut 14 to 20 µm sections in cryostat.
- Collect sections into SuperFrost Plus Gold slides, avoid more than 3 sections per slide, and OCT overlapping with tissue from a previous section.
- Let sections dry at room temperature for 1 hour.
- Store sections at -80^{o}C for no more than 4-5 weeks.

Part 1 : Sample preparation for frozen **Nodose ganglia** samples

1 Select tissue – ~ 3 sections/sample.

2 Dry tissue at Room temperature for 01:00:00 to 02:00:00 . 3h
Cover the slide loosely with foil to avoid contaminations.

3 Wash in PBS 1X to remove OCT for 2X 00:15:00 . 15m

Part 1 : Sample preparation for **frozen brain** samples

4 Select tissue – ~ 3 sections/sample.

5 Wash in PBS 1X to remove agar or OCT for 2X 00:15:00 . 15m
Wash free-floating sections into net-wells and black tray.

6 Mount tissue on superfrost charged gold slides.

7 Dry at Room temperature for 01:00:00 - cover the slides loosely with foil to avoid contaminations. 1h

8 Rinse in ddH2O by moving slide rack up and down (3-5 times) for 3x 00:00:15 . 15s

Part 1 : Sample preparation

9 Dry at Room temperature for 01:00:00 - cover the slides loosely with foil to avoid contaminations. 1h

10 During this incubation, PRE-HEAT HYBRIDIZATION OVEN and SLIDE TRAY at 60 °C .

11 Bake at 60 °C for 01:00:00 . 1h

12 During this incubation, prepare 4% PFA and keep in the fridge 4 °C .

13 Post-fix in 4% PFA at 4 °C for 01:00:00 (in the fridge). 1h

14 During this time prepare the following buffers:

14.1 Make **1X target retrieval buffer**
RNAscope Target Retrieval (x10) **ACD Catalog #**322001 .
(Warm stock solution 10X in water bath at 40°C for ~10 min before diluting / 1 mL 10X stock solution + 9 mL ddH_20)

14.2 Make **1X wash buffer** (provided in
RNAscope Fluorescent Multiplex Reagent Kit **ACD Catalog #**320850).
- Warm 50X stock solution in water bath at 40°C for 10-20 min before diluting
- 1mL 50X stock solution + 49 mL ddH_20. MIX WELL!!!
- 3L of 1X wash buffer -> 60 mL 50x stock solution (1 bottle) + 2.94 L ddH_20
- Store 1X wash buffer at RT for up to 1 month.

14.3 Prepare bottles with EtOH gradient solutions: 50%, 70%, and 100%.

Part 2: RNAscope pre-treatments 2h 8m 45s

15 ***NOTE** – After each step (most important after washes)- Remove excess liquid from the slides before adding new reagents by tapping or gently flicking the slide on absorbent paper.
If you have a hydrophobic barrier, you can also use a Kimwipe to absorb the left-over liquid. Tilting the slide slightly and allowing solutions to pool in one corner on the hydrophobic barrier to then dab with a Kimwipe works well.

Dehydration steps using EtOH gradient: 50%, 70%, 100%, new 100% - at
Room temperature , 5 min/each.

16 TURN ON STEAMER and SETUP at 100 °C . Use thermometer to check temperature.

17 Dry at 60 °C for 00:15:00 . 15m

18 Add **RNAscope hydrogen peroxide** - incubate slides at Room temperature for 00:10:00 . 10m

19 If active bubbling happens, please extend the Room temperature incubation to 00:20:00 . 20m

20 15. Rinse in ddH2O by moving slide rack up and down (3-5 times) for 2 x 00:00:15 . 15s

21 Dry at 60 °C for 00:15:00 . 15m

22 PLACE JARS WITH ddH2O AND TARGET RETRIEVAL (TR) SOLUTION INTO STEAMER 00:15:00 before is needed. Before starting the target retrieval step make sure that the TR buffer is not actively bubbling. 15m

23 TR step in the steamer -> place slides in ddH2O jar for 15 sec then move them into TR solution jar for 00:10:00 100 °C . 10m

24 Rinse in ddH2O 00:00:15 by moving slide rack up and down (3-5 times). 15s

25 Dip in 100% EtOH at Room temperature for 00:03:00 . 3m

26 Dry 60 °C for 00:10:00 . 10m

27 **Draw hydrophobic barrier** around the tissue:
Using the hydrophobic pen,
ImmEdge hydrophobic barrier pap pen **Vector Laboratories Catalog #**H-4000 , make a barrier as close to tissue as possible (without touching it) to reduce the amount of solution needed to cover the surface area.

While waiting for pen to dry, PRE-HEAT OVEN and SLIDE TRAY AT 40 °C .

28 Add (4-6) drops of **protease III** (provided in
RNAscope Fluorescent Multiplex Reagent Kit **ACD Catalog #**320850
RNAscope Protease III **ACD Catalog #**322337) , digest in humidity chamber at
40 °C for 00:30:00 . 30m

29 During protease incubation:

29.1 **Equilibrate amplification reagents** (provided in RNAscope Fluorescent Multiplex Reagent Kit **ACD Catalog #**320850) :
Remove AMP1, AMP2, AMP3, HPR-C1, HPR-C2, HPR-C3, HPR blockers from the fridge and equilibrate at RT.

29.2 **Prepare the probes** (provided in RNAscope Fluorescent Multiplex Reagent Kit **ACD Catalog #**320850) 10m
Warm probes and probe stock solution in water bath at 40 °C for 00:10:00
cool at Room temperature
Briefly spin probe stock solutions
Mix probes into a new tube - Probe ratios = C2:C3:C4:C1 = 1:1:1:50.
Example: 4µL C3 + 4µL C2 + 192 µL C1
If not using a C1 probe, use **RNAScope probe diluent** in its place
Invert tube several times to mix before use.
Store mixed probes at 2 °C - 8 °C for up to 6 months.

30 Rinse in ddH2O by moving slide rack up and down (3-5 times) for 2 x 00:00:15 . 15s

31 After this step, proceed IMMEDIATELLY to the RNAscope assay.

Part 3: RNAscope assay 3h 42m

32 PROBE HYBRIDIZATION 2h
Add (4-6) drops of probe mixture to appropriate slide(s), add positive probe to positive control slide, add negative probe to negative control slide. Incubate at 40 °C for 02:00:00 .

33 During this time prepare 5x SSC buffer:
1.25mL 20x SSC + 3.75mL ddH20
5x SSC buffer can be stored for up to 2 months at RT

34 Rinse with 1X wash buffer for 2x 00:02:00 . 2m

35 Cover tissue with 5x SSC, incubate at Room temperature Overnight in covered humidified chamber. 2m

36 **DAY 2**

TURN ON HYBRIDIZATION OVEN and setup it at 40 °C .

37 Remove 5x SSC solution.

38 Rinse with 1X wash buffer at Room temperature for 00:02:00 . 2m

39 Add drops of Amp #1 at 40 °C for 00:30:00 (in humidified chamber into hybridization oven). 30m

40 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

41 Add drops of Amp #2 at 40 °C for 3 00:30:00 . 30m

42 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

43 Add drops of Amp #3 at 40 °C for 00:15:00 . 15m

44 During this time, dilute **Opal fluorophores** (range 1:750-1:3K**; usually dil. 1:1500) in TSA buffer**:

44.1 Determine volume needed (150-220 ul/slide).
Example: 0.5µL fluorophore in 750µL TSA buffer from kit

44.2 Use tinfoil to cover tubes with the solutions as fluorophores are light sensitive.

NOTE: if Opal 690 is assigned to C1 or C2, its concentration may need to be increased.

45 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

46 Add drops of HRP-C1 at 40 °C for 00:15:00 . 15m

47 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

48 Add drops of diluted Opal selected for C1 at 40 °C for 00:30:00 . 30m

49 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

50 Add drops of HRP-blocker at 40 °C for 00:15:00 . 15m

51 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

52 Add drops of HRP-C2 at 40 °C for 00:15:00 . 15m

53 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

54 Add drops of diluted Opal selected for C2 at 40 °C for 00:30:00 . 30m

55 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

56 Add drops of HRP-blocker at 40 °C for 00:15:00 . 15m

57 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

58 Add drops of HRP-C3 at 40 °C for 00:15:00 . 15m

59 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

60 Add drops of diluted Opal selected for C3 at 40 °C for 00:30:00 . 30m

61 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

62 Add drops of HRP-blocker at 40 °C for 00:15:00 . 15m

63 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

64 Add drops of HRP-C4 at 40 °C for 00:15:00 . 15m

65 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

66 Add drops of diluted Opal selected for C4 at at 40 °C for 00:30:00 . 30m

67 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

68 Add drops of HRP-blocker at 40 °C for 00:15:00 . 15m

69 Rinse with 1X wash buffer at Room temperature for 2x 00:02:00 . 2m

70 DAPI solution from RNAscope kit at Room temperature for 00:00:30 . 30s

71 Air dry slides briefly, covered (slides are light sensitive).

72 Coverslip with ProLong Gold Antifade Mountant (from Thermo Fisher) or other comparable fluorescent mountant.

73 Immediately store slides in a light-proof box at 4 °C Dry 30 min to O/N.

73.1 Do not leave out at RT.

73.2 To prolong the signal, clear nail polish may be used to seal the coverslip to the slide.

73.3 Image slides after 8 hours or within 2 weeks. Signal deteriorates fast.