RNAScope v2 Protocol for Fixed-Frozen Cochlear Sections

Acronyms or shorthands:

DEPC diethyl pyrocarbonate
EDTA ethylene diamine tetraacetic acid
Fab AffiniPure Fab Fragment Donkey Anti-Mouse IgG
Neg Neg-50 (a colorless water-soluble frozen section medium)
PBS phosphate buffered salts
PFA paraformaldehyde
RT room temperature

Recipes:

4% PFA in 1xPBS: 10mL 16% PFA + 4mL 10xPBS, up to 40 mL total volume with DEPC-H_2O
120 mM EDTA in 1xPBS: 240mL 0.5M EDTA + 100mL 10xPBS, up to 1L total volume with DEPC-H_2O
10% sucrose in 1xPBS: 100g sucrose + 100mL 10xPBS, up to 1L total volume with DEPC-H_2O
30% sucrose in 1xPBS: 300 g sucrose + 100mL 10xPBS, up to 1L total volume with DEPC-H_2O
60% sucrose in 1xPBS: 600g sucrose + 100mL 10xPBS, up to 1L total volume with DEPC-H_2O
0.02% PBST: 20µL Triton-x + 10mL 10xPBS, up to 100mL total volume with DEPC-H_2O
0.5% PBST: 0.5mL Triton-x + 10mL 10xPBS, up to 100mL total volume with DEPC-H_2O

Best practices:

- Prepare all solutions in RNase-free or DEPC-treated water.
- All wash steps are done in a slide tray.
- Apply all solutions, except Fluoromount G, gently near the corners & edges of the hydrophobic barrier.
- Remove solutions with kimwipes, rather than aspiration.

Preparing adult fixed-frozen cochlear sections for RNAscope:

1. Perfuse adult mouse with 4% PFA in 1xPBS.
2. Quickly extract inner ear from temporal bone and prick bony wall close to the basal turn.
3. Fix ear at **RT** for **2 hr**.
4. Wash with 1xPBS.
5. Decalcify ear at **4°C** for **48h** in 120 mM EDTA in 1xPBS.
6. Wash with 1xPBS.
7. Apply 10% sucrose in 1xPBS. Rotate at **4°C** for **30 min**, or until ear sinks to bottom of tube.
8. Apply 30% sucrose in 1xPBS. Rotate at **4°C** for **2 hr**, or until ear sinks to bottom of tube.
9. Apply 1:1 Neg and 60% sucrose in 1xPBS. Rotate at **4°C**, **overnight**.
10. Apply Neg only. Rotate at **4°C** for **2 hr**.
11. Embed ear with fresh Neg in a disposable base mold (7x7x5 mm).
12. Collect 9 duplicate slides of 20-micron cochlear sections on slides that were coated twice with Poly L Lysine.
13. Dry slides at RT for 3-14 hours and then store in -80°C.

Day 1 of RNAscope v2

Preparation:

1. Wet both HybEZ oven tray and slide-holding tray. Bring slides to RT for **10 min**, covered in a slide holding tray.
2. Set HybEZ oven to 40°C.
3. Bring Protease III, Multiplex v2 kit, and probes to RT.
4. Dilute Opal dyes in TSA buffer (1:1500; 150-250 µL per slide); dilutions can be kept at 4°C in darkness for up to one month. Keep Opal dilutions in darkness as much as possible.

5. Before preparing the probe mix (150-250 µL per slide), heat C1 probe or probe diluent to dissolve precipitate.
6. Heat 50xWash Buffer in 37°C to dissolve precipitate before preparing 1X Wash Buffer.

Post-Fixation:

1. Draw hydrophobic barrier tightly around the tissues. Wash Neg off with 1XPBS at **RT**, **10 min**.
2. Apply fresh 4% PFA in 1XPBS at **RT**, **10 min**.
3. Wash with 1XPBS at **RT** for **5 min**, twice.

Protease Treatment:

1. Place slides on HybEZ oven tray. Apply Protease III (4-6 drops). Incubate at **40°C** for **1 hr**.
2. Remove slides and wash with 1XPBS at **RT**, **10 min**.

Probe Hybridization:

1. Apply probe mix (C2/C3 probes 1:50 in C1 probe/probe diluent). Incubate at **40°C** for **2 hr**.
2. Wash with 1xWash Buffer at **RT** for **5 min,** twice.

Amplification:

4-6 drops of each reagent are usually enough to cover the tissues. Tilt the slide to spread the solution. All washes are with 1xWash Buffer.

1. Apply **v2 Amp 1**. Incubate at **40°C** for **30 min**. Wash at **RT** for **5 min**, twice.
2. Apply **v2 Amp 2**. Incubate in **40°C** for **30 min**. Wash at **RT** for **5 min,** twice.
3. Apply **v2 Amp 3**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.
4. *If you used a C1 probe*,
    a. Apply **HRP-C1**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.
    b. Apply **Opal** dilution [light sensitive]. Incubate in **40°C** for **30 min**. Wash at **RT** for **5 min**, twice.
    c. Apply **HRP blocker**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.
5. *If you used a C2 probe*,
    a. Apply **HRP-C2**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.
    b. Apply **Opal** dilution [light sensitive]. Incubate in **40°C** for **30 min**. Wash at **RT** for **5 min**, twice.
    c. Apply **HRP blocker**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.
6. *If you used a C3 probe*,
    a. Apply **HRP-C3**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.
    b. Apply **Opal** dilution [light sensitive]. Incubate in **40°C** for **30 min**. Wash at **RT** for **5 min**, twice.
    c. Apply **HRP blocker**. Incubate in **40°C** for **15 min**. Wash at **RT** for **5 min**, twice.

Immunohistochemistry:

1. Block with 10% normal serum in 0.5% PBST at **RT**, **30 min** to **1 hr**.
2. <u>If planning to use a mouse antibody</u>, block with 1:50 Fab in 0.02% PBST at **RT** for **30 min** to **1 hr**. Wash with 0.02% PBST at **RT** for **5 min**, twice.
3. Apply primary antibody mix at **4°C**, **overnight**. Wash with 1XPBS at **RT** for **20 min**, thrice.

<u>Day 2</u>

4. Apply secondary antibody mix at **RT** for **2 hr**. Wash slides in 1XPBS at **RT** for **20 min**, thrice.

Mount slides with Fluoromount G. Store in covered tray overnight at RT. Store in 4°C in the long term.