Immunohistochemistry 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
1M sodium citrate buffer: 294 g tri-sodium citrate, adjust pH to 6.0, up to 1L total volume with Milli-Q water
10mM sodium citrate buffer: 10 mL 1M buffer + 0.5mL Tween 20, adjust pH to 6.0, up to 1L total volume with Milli-Q water

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.

Pretreatment with Heat-Induced Antigen Retrieval (*Optional*)**:**

1. Remove slides from -80°C. Wet slide holder towels with ddH_2O.
2. Demarcate tissues on slide with pap pen.
3. Equilibrate slides to **RT**, **10-15 min**. Keep slides protected from light whenever possible.
4. Wash slides in 1xPBS at **RT**, **10 min**.
5. Fix with 4%PFA in 1xPBS at **RT**, **10 min**.

6. Wash slides in 1xPBS at **RT** for **5 min**, twice.
7. Bring ~300 mL of AR buffer (10 mM sodium citrate buffer) to a boil in the microwave. Cover the flask with Saran wrap before placing it in the microwave.
8. As the buffer approaches boiling, quickly transfer slides into the metal holder.
9. Quickly pour the boiling buffer into a thermos and add the metal holder with slides into the thermos.
10. Place the thermos into Styrofoam box. Incubate for **10-20 min**.
11. Quickly remove the metal slide holder. Transfer hot buffer to white citrate slide holder. Quickly transfer slides from metal slide holder to white slide holder. Incubate slides in citrate holder with a loose cap. Allow to cool down in **RT** for **15 min**.
12. Wash slides in 1XPBS at **RT** for **5 min**, twice.

Blocking/Permeabilization

1. Apply blocking solution of 10% Normal Donkey Serum (NDS)[1] in 0.5% Triton-x in 1XPBS (PBST) at **RT**, **1 hr**.
2. *If planning to use Fab*[2], add these steps: Wash slides in 0.02% PBST at **RT** for **5 min**, twice**.** Apply 1:10 dilution of Fab Donkey Anti-mouse IgG in 0.5% PBST at **RT**, **30 min-1 hr**.

Staining

3. Wash slides in 0.02% PBST at **RT** for **10 min**, thrice.
4. Apply primary antibody solution[3] at **4°C**, **overnight** or at **RT**, **2 hr**.
5. Wash slides in 1XPBS for **1 hr**, once every **20 min**.
6. Apply secondary antibody solution at **RT**, **2 hr**.
7. Wash slides in 1XPBS for **1 hr**, once every **20 min**.
8. Mount with Fluoromount G.

[1] Use the serum specific to the animals that your primary antibodies were prepared in.
[2] Blocking with fab fragments reduces nonspecific binding for primary antibodies made in mice.
[3] Antibodies are diluted in 0.02% PBST. Dilution factors are specific to each primary antibody and usually halved when incubating overnight. Secondary antibodies are diluted at 1:300.