RNAscope Protocol Multiplex Fluorescent v2 (Quick version EA)

(To use with kit Cat No. 323100 or 323110)

Notes: this protocol works for both retinal tissue (E14-P11 tested) and embryonic spinal cords under PFA fixation conditions.

Acronyms and shorthands:

PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
DEPC	Diethyl Pyrocarbonate
RT	Room temperature
O/N	Over night

Recipes:

4% PFA in 1xPBS: 10mL 16% PFA + 4mL 10x PBS, up to 40mL total volume with DEPC H_2O

Day1

Preparing

1. Bring HybEZ Oven to **40°C**. Wet the bottom filter paper with water and pre-warm tray for at least **20 MIN**
2. Equilibrate the slides @ **RT** for **30 MIN** before processing the sections.
3. Prepare 3 L of 1X wash buffer by adding 2.94 L distilled water to 1 bottle (60 mL) 50X wash buffer (If precipitation occurs, **warm it up @ 40°C for 10–20 MIN** before making 1X wash buffer)
4. Warm probes for **10 MIN** @ **40°C** in a water bath or incubator, then cool to RT
5. Mix **1:1:50** ratios of C2, C3, and C1 probes (150-200µL per slide). Invert several times (Can be stored @ 4°C for up to 6 months)
6. When the slides have equilibrated to RT, draw a hydrophobic barrier around the sections. Let dry
7. Wash the slides with 200 mL 1X PBS in a Tissue-Tek slide rack for **5 MIN** to remove the OCT/NEG-50.
8. Postfix slides by immersing in pre-chilled 4% DEPC-PFA for **15mins** at **4°C**. Rinse 2 times with DEPC-PBS

Hydrogen Peroxide treatment

1. Lay slides on the bench and add ~5-8 drops of RNAsope Hydrogen Peroxide to cover the entire section.
2. Incubate slides for **10min** at **RT**.
3. Remove Hydrogen Peroxide from one slide at a time by tapping the slide on absorbent paper. Immediately submerge in DEPC-water.
4. Wash slides 3-5 times by moving rack up and down in the DEPC-water.
5. Repeat with fresh DEPC-water.

Protease treatment

1. Apply RNAscope **Protease III** (3-4 drops) to permeabilize the tissue.
2. Incubate for **10 MIN @ 40°C.** (these conditions have been optimized for 20µm sections and further IHC)
3. Briefly submerge in 200ml of 1x PBS to remove excess protease.
4. Repeat with fresh PBS.

(1.5h)

Probe Hybridization

1. Add **~150-200ul** of appropriate probe to each slide. Incubate for **2 hours @ 40°C**.
2. Remove AMP1, AMP2, AMP3, HRP-C1 (if using), HRP-C2 (if using), HRP-C3 (if using) and HRP blockers from the refrigerator and place at RT during this step.
3. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
4. Repeat with fresh 1x wash buffer.
5. OPTIONAL STOPPING POINT→ store slides in 5X SSC overnight at RT. Then was with 1x wash buffer for 2min at RT.

(2h)

Signal Amplification: AMP 1

1. **Add ~4 drops of AMP 1-FLv2** to cover each section. Incubate for **30 MIN @ 40°C**
2. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
3. Repeat with fresh 1x wash buffer.

Signal Amplification: AMP 2

1. **Add ~4 drops of AMP 2-FLv2** to cover each section. Incubate for **30 MIN @ 40°C**
2. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
3. Repeat with fresh 1x wash buffer.

Signal Amplification: AMP 3

1. **Add ~4 drops of AMP 3-FLv2** to cover each section. Incubate for **15 MIN @ 40°C**
2. ****During this time prepare Fluorophores****
3. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
4. Repeat with fresh 1x wash buffer.

******Prepare Opal™ dye******

Prepare only the fluorophores you will be using. If using one probe, prepare only one fluorophore.

1. Reconstitute Opal Dye following the manufacturer´s instructions (Akoya Bioscience)
2. Calculate the final volume needed per fluorophore
3. Dilute Opal dye using the TSA buffer provided by RNAscope kit v2.
4. Store diluted Opal dye up to 1 month at 4°C in the dark.

Fluorophore	Dilution	No. of slides	Total TSA buffer (μL)	Dye (μL)
Opal 520	**1:1,500**			
Opal 570	**1:1,500**			
Opal 690	**1:1,500**			

Develop: HRP-C1 signal

if using only C2 or C3 probes, skip this and go to HRP-C2 and/or HRP-C3 steps

1. **Add ~4 drops of HRP-C1** to cover each section. Incubate for **15 MIN @ 40°C**
2. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
3. Repeat with fresh 1x wash buffer.
4. Add 150μl-200μl of diluted **Opal dye** to cover each slide. Incubate for **30 MIN @ 40°** (channels and fluorophores can be mixed and matched but do not assign the same fluorophore to more than one channel)
5. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
6. Repeat with fresh 1x wash buffer.
7. **Add ~4 drops of HRP blocker** to cover each section. Incubate for **15 MIN @ 40°C**

8. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
9. Repeat with fresh 1x wash buffer.
10. Stop here and proceed with immuno or mount with Fluoromount-G if you are just using probe C1.

(2.5h)

Develop: HRP-C2 signal

if using C3 probe and no C2 probe, skip this and go to HRP-C3 step

1. **Add ~4 drops of HRP-C2** to cover each section. Incubate for **15 MIN @ 40°C**
2. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
3. Repeat with fresh 1x wash buffer.
4. Add 150µl-200µl of diluted **Opal dye** to cover each slide. Incubate for **30 MIN @ 40°** (channels and fluorophores can be mixed and matched but do not assign the same fluorophore to more than one channel)
5. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
6. Repeat with fresh 1x wash buffer.
7. **Add ~4 drops of HRP blocker** to cover each section. Incubate for **15 MIN @ 40°C**
8. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
9. Repeat with fresh 1x wash buffer.
10. Stop here and proceed with immuno or mount with Fluoromount-G if you are not using a probe in C3.

(1h)

Develop: HRP-C3 signal

1. **Add ~4 drops of HRP-C3** to cover each section. Incubate for **15 MIN @ 40°C**
2. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
3. Repeat with fresh 1x wash buffer.
4. Add 150µl-200µl of diluted **Opal dye** to cover each slide. Incubate for **30 MIN @ 40°** (channels and fluorophores can be mixed and matched but do not assign the same fluorophore to more than one channel)
5. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
6. Repeat with fresh 1x wash buffer.
7. **Add ~4 drops of HRP blocker** to cover each section. Incubate for **15 MIN @ 40°C**
8. Submerge the slides in 1x wash buffer for **2 MIN @ RT**.
9. Repeat with fresh 1x wash buffer.
10. Proceed with IHC or mount with Fluoromount-G.

(1h)

OPTIONAL: RNAscope + IHC (Immunostaining)

1. After the HRP blocker and the final wash, rinse the slides briefly with 1x PBS.
2. Block the slides in blocking buffer 0.5% Triton X-100, 5% normal Donkey serum in PBS **@ RT for 1 hour**.
3. Primary antibody incubation @ **4°C O/N**

Day 2

4. Wash 5x with 1X PBS, **5 min** each time at **RT**
5. Secondary antibody incubation @ **RT** for **1-2 hours**
6. Wash 5x with 1X PBS, **5 min** each time
7. Mount slides with DAPI-fluoromount-G.