**RNAscope performed on whole-mounts of *Drosophila* and**

***Aedes aegypti* proboscis and antenna**

**Step A. Fix the tissue (overnight 16 hours)**

1) Dissect either 10 proboscises or 10 antennae into PBS.

2) Wash once with PBS.

3) Fix in 4% paraformaldehyde in PBS (PFA) for 16 hours at 4 °C.

**Note:** Prepare 4% PFA in 1X PBS. Use FRESH fixatives. Do NOT reuse.

4) Wash tissue with 1X PBS 3 – 5 times.

**Step B. Dehydrate the sections (30 min)**

1. Prepare 10 ml each of 50 % ethanol, 70 % ethanol, and 100 % ethanol in 15 ml tubes.
2. Dehydrate tissue in 50 % ethanol for 5 min at RT.
3. Dehydrate tissue in 70 % ethanol for 5 min at RT.
4. Dehydrate tissue in 100 % ethanol for 5 min at RT.
5. Remove the ethanol and let the sample dry completely for 30 minutes at RT.

**Step C. Permeabilization and Target retrieval (15 min)**

1. Remove the ethanol and let the sample dry completely for 30 minutes at RT
2. Add ~2-3 drops of RNAscope Hydrogen Peroxide to cover the sample.
3. Incubate slides for 10 MIN at RT.
4. Remove RNAscope Hydrogen Peroxide solution and wash the tissue with distilled water 3–5 times.
5. Add 500ul of 1X RNA retrieval reagent to cover the sample.
6. Incubate the sample for 5min at 99 degrees heating block.
7. Remove RNA retrieval reagent solution and wash the tissue with distilled water 3–5 times.
8. Add 100 % ethanol for 3 min at RT
9. Remove the ethanol and let the sample dry completely for 30 minutes at RT.

**Step D. Protease treatment (30 min)**

1. Add 2~3 drops of RNAscope Protease reagent to entirely cover the sample.
2. Incubate at 40°C for 30 min
3. Remove RNAscope Protease solution and wash the tissue with distilled water 3–5 times.

**Step E. RNAscope® Assay (4 hrs)**

1. Ensure that the probes are prewarmed and cooled to RT prior to use.
2. Add 100-200ul of the appropriate probe to entirely cover each sample and incubate for 4 hours at 40 °C.
3. Remove RNAscope Probes and wash the tissue with 1x wash buffer 3–5 times.

**Step F. Hybridize RNAscope® HiPlex Amp 1 (2 hrs)**

1. Add 100-200ul of RNAscope HiPlex Amp 1 to entirely cover each sample and incubate for 2 hours at 40 °C.
2. Remove RNAscope HiPlex Amp 1 and wash the tissue with 1x wash buffer 3–5 times.

**Step G. Hybridize RNAscope® HiPlex Amp 2 (2 hrs)**

1. Add 100-200ul of RNAscope HiPlex Amp 2 to entirely cover each sample and incubate for 2 hours at 40 °C.
2. Remove RNAscope HiPlex Amp 2 and wash the tissue with 1x wash buffer 3–5 times.

**Step H. Hybridize RNAscope® HiPlex Amp 3 (1 hrs)**

1. Add 100-200ul of RNAscope HiPlex Amp 3 to entirely cover each sample and incubate for 1 hours at 40 °C.
2. Remove RNAscope HiPlex Amp 3 and wash the tissue with 1x wash buffer 3–5 times.

### **Step I. Develop HRP-C1 signal (2 hrs)**

1. Add 100-200ul of RNAscope HRP-C1 to entirely cover each sample and incubate for 2 hours at 40 °C.
2. Remove RNAscope HRP-C1 and wash the tissue with 1x wash buffer 3–5 times.
3. Add 150–200 µL diluted Opal™ 520 to each slide and incubate for 2hr at 40°C.

(You can mix and match channels and fluorophores. For example, you may assign Opal™ 570 or Opal™ 690 to the C1 channel instead of Opal™ 520. If Opal™ 690 is assigned to the C1 channel, you may need to increase the concentration of Opal™ 690. Do not assign the same fluorophore to more than one channel.)

1. Remove Opal™ 520 and wash the tissue with 1x wash buffer 3–5 times.
2. Add 4–6 drops RNAscopeMultiplex FL v2 HRP blocker to entirely cover each sample.
3. Incubate for 30 min at 40 °C and wash the tissue with 1x wash buffer 3–5 times.

STOP HERE IF YOU ARE USING JUST ONE C1 PROBE. **Continue to Counterstain and mount the slides section**

Continue with HRP-C2 same as above.

If you are using the C3 probe instead of C2, use RNAscope Multiplex FL v2 HRP-C3 instead of HRP-C2.

**Continue to Counterstain and mount the sample Image the slides after eight hours and within two weeks.**