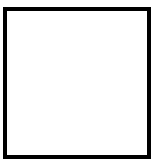
RNAscope Protocol ver.1

Pre-Assay Tissue Preparation

1. Slicing fresh frozen tissue
    a. Freeze specimen with dry ice or liquid nitrogen
        i. (adaptation: using isopentane)
    b. Cut 10-20 µM sections and mount onto Superfrost Plus Slides
        i. (adaptation: cutting at 16 µM, using StellarSci Slides)
    c. Keep sections at -20ºC to dry
    d. Store the slide box (wrapped air tight) **in a ziplock bag at -80ºC**
        i. Sections can be stored for 3 months prior to use **(Optional stop point)**
2. Pre-assay tissue processing
    a. Reagents needed: 4% NBF in 1x PBS (chilled), 200 mL 50% Etoh (RT), 200 mL 70% Etoh (RT), 600mL 100% Etoh (RT) – Etoh made with 1X PBS
    b. Pre-chill 200mL of 10% Normal buffered formalin or 4% PFA (in 1x PBS) in a tissue tek staining dish at 4ºC
        i. (adaptation: using 10% Neutral Buffered Formalin)
            1. Contains methanol which helps to permeabilize cells
    c. Remove slides from -80ºC, place in slide holder, and immediately **immerse in staining dish with 4% PFA in 1X PBS for 15min at 4ºC**.
    d. Place slide holder in staining dish with **50% Etoh at RT for 5min**
    e. Place slide holder in staining dish with **70% Etoh at RT for 5min**
    f. Place slide holder in staining dish with **100% Etoh at RT for 5min**
    g. (Repeat 2f) Perform another incubation in **100% Etoh at RT for 5 min**.
    h. Remove slides and place on absorbent paper (section facing up) **and air dry for 5 min**.
    i. Draw a barrier with the ImmEdge hydrophobic barrier pen. (0.75" x 0.75") – use template on next page (1 template per slide)
        i. **place image below underneath slide**)



    j. Let the barrier dry completely ~1 min.
3. Pre-treatment processing
    a. **Turn on hybridization oven and set to 40ºC** (also place a sheet of humidifying paper on the control tray and wet with distilled water).
    b. At RT, place the dried slides on the HybEZ slide rack, add **2-4 drops of pretreat 4 per section** and **incubate for 30 min at RT**.
        i. NOTE: this step may need to be adjusted between 15-30 min. depending on assay starting at 30 min
        ii. for chromogenic assay, this step is preceded by pretreat 1 for 10 minutes followed by a 1x rinse in dH2O

c. Remove liquid (by tapping or flicking) and immediately place slides in a tissue tek slide rack in a tissue tek staining dish filled with 1x PBS (move up and down 3-5 times)
d. (Repeat 3c) – **PROCEED IMMEDIATELY TO RNAscope ASSAY**
    i. Slides can stay in 1x PBS for upto 15 min.

(--Do not apply Pretreat 1 or Pretreat 2 to fresh, frozen sections when using the Fluorescent Multiplex Kit. Other sample types may need these treatments.)

4. *Fluorescent RNAscope assay*
    a. Notes: make sure to run positive (Ppb) and negative control probe (DapB) on target tissue sample with each assay (1 slide each)
    b. Prep chemicals and probes
        i. Wash Buffer
            1. 3L of wash buffer
                a. Warm 50x wash buffer to 40ºC for 10-20 min
                b. Mix 1 bottle of 50x was buffer (60mL) to 2.94L distilled water
        ii. Probes
            1. Warm probes (CRF C1, EGFP C3, Ppb, and DapB) **to 40ºC for 10 min, then cool to RT**
            2. Spin down C2 or C3 probes
            3. Mix probes at 1:1:50 (C2 or C3 to C1) – (may need to cut top of C1 probe to ensure correct amount)
                a. Mixed probes can be stored at 4ºC for up to 6 mos.
        iii. Bring AMP1-4FL to room temp
        iv. Hybridize probe
            1. Tap to remove excess liquid and add **~4 drops of appropriate probe** to slides
            2. Place in HybEZ oven tray and secure in oven. **Incubate for 2 HRS at 40ºC** (ensure turn lock on oven is secure to prevent evap)
            3. Remove slides 1 AT A TIME, tap to remove liquid, and immediately immerse in 1X wash buffer for 2 min at RT. Agitate slides by moving up and down.
            4. Repeat step 3
        v. Hybridize AMP1-FL
            1. Tap to remove excess liquid and add **~4 drops of AMP1-FL** to slides
            2. Place in HybEZ oven tray and secure in oven. **Incubate for 30 min at 40ºC** (ensure turn lock on oven is secure to prevent evap)
            3. Remove slides 1 AT A TIME, tap to remove liquid, and immediately immerse in **1X wash buffer for 2 min at RT. Agitate** slides by moving up and down.
            4. Repeat step 3
        vi. Hybridize AMP2-FL
            1. Tap to remove excess liquid and add **~4 drops of AMP2-FL** to slides

2. Place in HybEZ oven tray and secure in oven. **Incubate for 15 min at 40ºC** (ensure turn lock on oven is secure to prevent evap)
3. Remove slides 1 AT A TIME, tap to remove liquid, and immediately immerse in **1X wash buffer for 2 min at RT. Agitate** slides by moving up and down.
4. Repeat step 3

vii. Hybridize AMP3-FL
1. Tap to remove excess liquid and add **~4 drops of AMP3-FL** to slides
2. Place in HybEZ oven tray and secure in oven. **Incubate for 30 min at 40ºC** (ensure turn lock on oven is secure to prevent evap)
3. Remove slides 1 AT A TIME, tap to remove liquid, and immediately immerse in **1X wash buffer for 2 min at RT. Agitate** slides by moving up and down.
4. Repeat step 3

viii. Hybridize AMP4-FL
1. Tap to remove excess liquid and add **~4 drops of AMP4 ALT B-FL** to slides
    a. Alt-B has C1 at Atto 550nm and C3 at Atto 647nm excitation
2. Place in HybEZ oven tray and secure in oven. **Incubate for 15 min at 40ºC** (ensure turn lock on oven is secure to prevent evap)
3. Remove slides 1 AT A TIME, tap to remove liquid, and immediately immerse in **1X wash buffer for 2 min at RT. Agitate** slides by moving up and down.
4. Repeat step 3

ix. Counterstain and Mount
1. Tap to remove excess liquid and add **~4 drops of DAPI** to slides
2. Place in HybEZ oven tray and secure in oven. **Incubate for 30 sec at RT**
3. Remove DAPI from slides and immediately add Prolong Gold Antifade (without Dapi) and coverslip
4. **Store slides in dark at 4ºC**