IF-ISH HCR super combo, 5-10um paraffin sections (Gillis)

Day 1

Before you begin…

-heat one water bath to 60°C, and a second water bath to 95°C
-RNaseZAP™ & dry one glass coplin jar and two pink top-tubes

Rehydrate sections

Histosol – 5 minutes
Histosol – 5 minutes
100% EtOH – 2 minutes
100% EtOH – 2 minutes
95% EtOH/DEPC PBS – 3 minutes
70% EtOH/DEPC PBS – 3 minutes
50% EtOH/DEPC PBS – 3 minutes
DEPC water – 3 minutes

Antigen retrieval

1. Warm slides to 60°C in preheated DEPC water for 5 minutes (RNase-free pink top tube).

2. Transfer warmed slides to pre-warmed antigen retrieval solution in second RNase-free pink top tube (DEPC 10mM sodium citrate, pH6.0). Incubate for 25 minutes at 95°C (important: this step must be done in a plastic tube!).

3. Transfer pink top tube to -20°C freezer for ~15 minutes (just long enough to reach room temp – make sure it doesn't freeze).

4. Transfer slides to glass coplin jar. Rinse 2 x 2 minutes in DEPC PBS + 0.1% tween at room temp.

Block

Drain/blot slide edges and place in humidified chamber. Block with ~1-2 ml of antibody buffer per slide (cover all sections) for 1 hour at room temp.

1°AB

Dilute 1° antibodies in antibody buffer. Apply ~150-200 ul 1° antibody solution per slide. Coverslip with parafilm and place in humidified chamber overnight at 4°C.

Day 2

Before you begin…

-Prepare a humidified chamber (50% formamide/2X SSC)
-Pre-warm 2.5 ml of HCR hybridization buffer/slide at 37℃ (found in freezer, can return unused in 15 ml conical)

Intiator-labeled 2^{o} antibodies

1. Remove slides from humidified chamber and drain excess antibody solution.

2. Wash slides 3 x 5 minutes in DEPC PBS + 0.1% tween (RNase-free coplin).

3. During washes, dilute initiator-labeled 2^{o} antibodies to 1 ug/ml in ~200 ul of antibody buffer per slide (~0.5 ul 2^{o} per 200 ul buffer).

4. After washes, drain/blot slide edges and apply ~200 ul of 2^{o} antibody solution per slide. Coverslip with parafilm and incubate for 1 hour in humidified chamber at room temp.

Hybridisation

1. Remove slides from humidified chamber and drain excess 2^{o} solution.

2. Post-fix sections by placing slides in 4% PFA (32% EM PFA + DEPC PBS) for 10 minutes at room temp (pinky).

3. Wash slides 2 x 5 minutes in DEPC PBS + 0.1% tween (coplin).

4. Wash slides 1 x 5 minutes in 5x SSCT (coplin).

5. Drain excess solution from slides, and blot slide edges. Pre-hybridise slides by adding ~2 ml of pre-warmed hybridisation buffer/slide. Incubate for 30 minutes in humidified formamide chamber at 37^{o}C.

6. During pre-hybridisation step, dilute probe sets in pre-warmed hybridisation buffer. Add 0.8 ul of each 1 uM probe stock per 100 ul of hybridisation buffer (prepare ~300 ul of hybridisation buffer/slide, ~2.4 ul probe/slide). Return hybridisation buffer + probe mixture to 37^{o}C.

7. After 30-minute pre-hybridisation, remove humidified chamber from 37^{o}C incubator, drain off pre-hyb buffer and blot slide edges. Add 250 ul of hybridisation buffer + probe mixture to each slide, add glass coverslip and incubate overnight in humidified chamber at 37^{o}C.

Day 3

Before you begin…

-Prep humidified chamber (water); clean/reuse same from previous day
-Pre-warm 2.5 ml of amplification buffer/slide to room temp
-Pre-warm the following wash solutions to 37℃ in water bath:

- *HCR wash buffer (40 ml in coplin, put in before heating bath)*
- *75% wash buffer: 25% 5x SSCT (10 ml in pinky)*
- *50% wash buffer: 50% 5x SSCT (10 ml in conical)*
- *25% wash buffer: 75% 5x SSCT (10 ml in conical)*
- *5x SSCT (10 ml in conical)*

Amplification

1. Remove hybridised slides from humidified chamber, and wash briefly in pre-warmed HCR wash buffer to float off coverslip (coplin remains in 37°C bath). Then wash as follows (all in 37°C bath):
   a. 75% wash buffer: 25% 5x SSCT (15 min; pinky) **prep chamber*
   b. 50% wash buffer: 50% 5x SSCT (15 min; pinky)
   c. 25% wash buffer: 75% 5x SSCT (15 min; pinky)
   d. 5x SSCT (15 min; pinky) **turn on heat block*

2. Wash slides in 5X SSCT at room temp (5 minutes; coplin). **grab hairpins during this wash, thaw in dark*

3. Prep hairpins by separately heating 4 uL (per 100 uL of amplification buffer) of each hairpin stock to 95°C for 90 sec, then cooling at room temp (in dark) for 30 minutes. Prep enough hairpins to allow ~200 uL of amplification buffer + hairpins per slide (~full 8 uL aliquot per slide; quick vortex/spin before heat).

4. Pre-amplify by applying ~2.5 mL of room temp amplification buffer per slide, and incubate in humidified chamber for 30 minutes at room temp.

5. Add hairpins to an appropriate amount of amplification buffer and briefly vortex. Drain off pre-amplification buffer from slides, and apply 200 uL of amplification buffer + hairpins to each slide. Coverslip with parafilm and incubate slides in humidified chamber overnight at room temp (in dark). Give hairpins quick spin before pipetting into master mix.

Day 4

1. Remove parafilm coverslips and wash slides as follows (in dark):
    a. 5x SSCT (5 min; coplin)
    b. 5x SSCT (15 min; coplin)
    c. 5x SSCT (15 min; pinky)
    d. 5x SSCT (5 min; pinky)

2. Coverslip with Fluoromount G + DAPI. Two dime-sized drops: one at far end, other closer to slide middle. Gently lay coverslip beginning with end furthest from frosted panel. Dab excess fluoromount from edges. Keep in dark, and let sit for 24 h before imaging.