In situ hybridization by HCR on paraffin sections (Gillis Lab Protocol)

Day 1

Before you begin:

- *Prepare a humidified chamber (1:1 formamide/2X SSC)*
- *Pre-warm 2.5mL of HCR hybridization buffer/slide at 37℃*
- *Prepare and pre-warm 10ug/mL proteinase K in DEPC PBS at 37℃ (proteinase K treatment will be performed in a pink-top tube, so prepare and pre-warm ~10mL per 4 slides)*

1. Dewax slides in Histosol (2 x 5 min). **[in a coplin jar]**
2. Rehydrate slides in 100% EtOH (2 x 5 min), then 2 min each in 90%, 70%, 50% EtOH/DEPC PBS, DEPC water, DEPC PBS + 0.1% Tween. **[in a coplin jar]**
3. Treat slides with 10ug/mL proteinase K in DEPC PBS at 37°C (10 min). **[in a pink-top tube]**
4. Rinse in DEPC water at room temp (2 min) **[in a coplin jar]**
5. Move slides to a humidified chamber. Pre-hybridise slides by adding ~2mL of pre-warmed hybridisation buffer/slide (add hyb buffer generously, until the slide is covered but without buffer spilling over the edges). Incubate for 30 minutes in a humidified chamber at 37°C.
6. During the pre-hybridisation step, dilute probe sets in pre-warmed hybridization buffer. Add 0.8uL of each 1uM probe stock/100uL of hybridization buffer (we prepare 300uL of hybridization buffer/slide), and return hybridization buffer + probe mixture to 37°C.
7. After the 30-minute pre-hybridisation is complete, remove the humidified chamber from the 37°C incubator, drain off the pre-hyb buffer and blot the edges of the slide on tissue paper. Add 250uL of hybridization buffer + probe to each slide, add a glass coverslip and incubate slides in a humidified chamber at 37°C overnight.

Day 2

Before you begin:

- *Prepare a humidified chamber (water)*
- *Pre-warm the following wash solutions to 37℃ in water bath (12mL each):*
    - *HCR wash buffer*
    - *75% wash buffer: 25% 5x SSCT*
    - *50% wash buffer: 50% 5x SSCT*
    - *25% wash buffer: 75% 5x SSCT*

- *5x SSCT*

– *Pre-warm 2.5mL of amplification buffer/slide to room temp*

9. Remove hybridized slides from humidified chamber, and wash briefly in pre-warmed HCR wash buffer (to float off the coverslip). Then wash as follows (all washes at 37°C **in a pink-top tube**):
   a. 75% wash buffer: 25% 5x SSCT (15 min)
   b. 50% wash buffer: 50% 5x SSCT (15 min)
   c. 25% wash buffer: 75% 5x SSCT (15 min)
   d. 5x SSCT (15 min)

10. Wash slides in 5X SSCT at room temp (5 min) **[in a coplin jar]**

11. Prepare hairpins by separately heating 4uL (per 100uL of amplification buffer) of each hairpin stock to 95°C for 90 sec, then cooling at room temp (in the dark) for 30 minutes. Prepare enough hairpins to allow 200uL of amplification buffer + hairpins per slide (i.e. 8uL of h1 and h2, for each hairpin, per slide).

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

13. Mix hairpins and amplification buffer and briefly vortex. Drain off pre-amplification buffer from slides, blot the edges of the slide, and apply 200uL of amplification buffer + hairpins to each slide. Add a parafilm coverslip and incubate slides in humidified chamber overnight at room temp (in the dark).

Day 3

14. Wash slides as follows (in the dark) **[in a coplin jar]**:
   a. 5x SSCT (5 min)
   b. 5x SSCT (15 min)
   c. 5x SSCT (15 min)
   d. 5x SSCT (5 min)

15. Coverslip with Fluoromount G + DAPI.