*Washes (*O*) start with a rinse (*o*). Washes are with 1 mL for 5 minutes. Washes involving hybridization solution are 500 uL. ‘Jet-stream’ during washes. Successive washes are done under a running clock. After washes, remove waste from wells in 150 uL increments and with the same 200 uL tip. When washing with the same solution, do not change tips unless you contaminate them. Do not contaminate the pipettes with liquid!!!*

NOTE: *Let methanol waste evaporate in the hood or pour it down the drain and flush 1 min.*

**Day 0**

*Make sure the glass viewing dish is coated with gel slick (ask Thomas for help). Dissect P10 pupal wings (P10 = bright red eyes, shortened Malpighian tubules, before any greying of the body or wings - see scheme on last page) in dH2O at RT. Allow the wings to inflate (P10 wings cannot over-inflate) before placing them in PBT + 4% PFA on ice in a 2mL tube.*

1. Fix the wings for 30 minutes at RT O

*Fixation causes the proteins to cross-link*

1. Wash 2x with methanol o O O
2. Wash 2x with 100% ethanol (200 proof) o O O
3. Store wings at -20°C in 100% ethanol (200 proof) O

**Day 1 (approximately 8 hrs)**

*Use a black background with the dissecting scope to view wings in a glass viewing dish.*

*Measure out 3.5mL of hybridization solution per sample in advance in a 15 or 50 mL falcon tube for later use.*

1. Get wings from -20°C O

*Transfer the wings by pipette (using a cut 200 uL tip)*

2. Wash 3x with 100% ethanol O O O

3. Incubate for 30 minutes in 1:1 Xylenes:Ethanol (1 mL total) O

*incubate in the hood*

*do not cover*

*discard all xylenes-containing washes the hood*

1. Make sure the big incubator is set to 65°C O
2. Wash 5x with 100% ethanol (200 proof) o O O O O O
3. Wash 2x with methanol o O O
4. Wash 3x with PBT o O O O
5. Fix for 30 minutes in PBT + 4% PFA at RT *(Jet-stream every 5-10 minutes)* O

*Get ice* O

1. Wash 5x with PBT o O O O O O
2. Mix 1 mL PBT + 0.4 uL proteinase K [10 mg/mL] while on ice O

*Proteinase K is in PBS*

*Mix proteinase K well before taking out! And always keep proteinase K*

*on ice (bring the stock immediately back to the freezer)!*

12. Replace PBT with 1 mL proteinase K solution O

13. Incubate at RT for 1 hour (*Jet-stream every 10-15 minutes)* O

14. Rinse 2x with PBT o o

15. Wash 2x with PBT O O

16. Post-fix for 30 minutes in PBT + 4% PFA at RT (*Jet-stream every 5-10 minutes)* O

17. Wash 5x with PBT o O O O O O

18. Wash in 1:1 PBT:hybridization solution O

19. Wash 3x in hybridization solution at RT O O O

20. Turn on dry heating block at 80°C O

21. Prehybridize for 1 hour in hybridization solution at 65°C O

*Blocking occurs*

22. Prepare the probe in a 2 mL tube

*Always wear gloves when using probe and always keep on ice*

a. Dilute probe 1 uL:500 uL hybridization solution O

b. Heat 5 minutes in dry block at 80oC O

c. Put and keep probe on ice *(prevents secondary RNA structures)* O

23. Replace all hybridization solution with diluted probe (1:500) on ice O

*Use 200 uL pipette with cut tip to transfer wings to a 2 mL tube with probe* O

24. Incubate overnight at 65°C for >18 hours, **gently** swirl periodically O

*This is an optional stopping point (2-3 days max)*

**Day 2 (approximately 4 hrs)**

1. Turn on dry heating block at 65°C O

2. Pre-warm 2.5 mL of hybridization solution per sample at 65°C O

3. Transfer wings back to the glass viewing dish from the tube O

*Using a 200 uL pipette with a cut tip*

4. Rinse with pre-warmed hybridization solution o

5. Incubate at 65°C for 1 hour in hybridization solution O

6. Incubate 3x for 30 minutes in pre-warmed hybridization solution O O O

*Place 750 uL of hybridization solution per sample at RT* O

*Get Ice* O

7. Prepare 1.5 mL of 1:1 PBT:hybridization solution O

8. Wash 2x with 1:1 PBT: hybridization solution at RT (not pre-warmed!) o O O

9. Wash 5x with PBT o O O O O O

10. Prepare 1:6000 Roche α-DIG AP Fab Fragments O

1,200*μL PBT:0.2 uL Roche α-DIG AP Fab Fragments (****on ice!****) in a 2mL tube*

11. Put 300 uL of 1:6000 Roche α-DIG AP Fab Fragments in a 2 mL tube on ice O

12. Pipette the wings into the tube containing the antibody on ice O

13. Incubate overnight at 4°C in a 2 mL Eppendorf tube O

**Day 3 (Approximately 3 hrs [pattern c develop within 45 mins])**

1. Wash 5x with PBT o O O O O O

*During the last wash prepare the staining* ***buffer*** *(recipe below)* O

2. Wash 3x with staining buffer o O O O

*During the last wash prepare the staining* ***solution*** *(recipe below)* O

3. Replace last liquid with 0.75 mL of staining solution O

4. Incubate in the dark O

5. Check for the pattern every 30 minutes O

6. Stop staining after the pattern looks good: Wash 2x with staining buffer o O O

7. Take images with the dissecting scope in staining buffer O

**PBT (1 L):**

100 mL 10x PBS

900 mL H2O

1 mL Triton X-100

**PBT + 4% PFA (40 mL):** *(Store at 4°C for max. a month)*

4 mL 10 x PBS

10 mL 16% PFA (Paraformaldehyde in ampulls)

400 ul 10% Triton X-100

25.6 mL H2O

**PBT + Proteinase K (1.5 mL):** *(Make fresh)*

1.5 mL PBT

0.6 uL Proteinase K [10 mg/ml] (in PBS)

**Hybridization solution (200 mL):** *(Store at -20°C)*

100 mL Formamide

50 mL 20x SSC

*Set pH to 5.5 (check with color strips) and filter sterilize*

2 mL Salmon Sperm DNA [10 mg/mL]

20 mg Heparin

200 uL Tween 20

48 mL H2O

**Staining Buffer (50 mL):** *(Make fresh)*

1 mL 5 M NaCl

2.5 mL 1 M MgCl2

2.5 mL 2 M Tris pH 9.5

50 uL Tween 20

44 mL H2O

**Staining Solution if using Promega solutions (750 ul):** *(Make fresh, keep dark)*

400 ul Staining Buffer

2.8 uL NBT (Progema ready-made 50 mg/mL solution)\*\*

1.4 uL BCIP (Progema ready-made 50 mg/mL solution)\*\*\*



**Staining Solution if using Bio-Rad solutions (400 ul):** *(Make fresh, keep dark)*

400 ul Staining Buffer

2.8 uL NBT (Bio-Rad 50 mg/mL solution made from powder, stored in Falcon tube)\*\*

5.6 uL BCIP (Bio-Rad 12.5 mg/mL solution made from powder, stored in Falcon tube)\*\*\*

**Basic solutions**

**100 mL of 2 M Tris pH 9.5:** 24.2 g Tris Base

**100 mL of 1 M MgCl2:** 20.3 g MgCl2 \* 6 H2O

**100 mL of 5 M NaCl:** 29.2 g NaCl

**20x SSC** see Maniatis

\*\*NBT is stored in the freezer! \*\*NBT is toxic in its powder form!

\*\*\*BCIP is stored in the fridge!

