Hackam Lab for Pediatric Surgical, Translational and Regenerative Medicine
Edward D. Miller Research Building
733 North Broadway, Room 471
Baltimore, MD 21287
410-955-9012

JOHNS HOPKINS MEDICINE

Hackam Lab
Whole Mount Protocol for Intestine, Lung, Liver and Brain (Embryo and postnatal tissues)

******Keep plate wrapped with foil through all steps if tissues are fluorescent labeled******

1. Harvest tissues, mark outer surface with INIDA INK before fixing in 4% Paraformaldehyde (PFA)

   (*For intestine—keep the intestine on paper towel, add a drop of INDIA INK, wait for couple of seconds, remove/dry excess INDIA INK, cut intestine longitudinally to open the lumen).*

2. Place tissues in wells (24-well plate) containing ~1mL 4%PFA, and fix at 4^{o}C (on rotator) for 2 hours.

3. Remove PFA, refill each well with 1 ml 1x PBS, allow washing on rotator for 10 minutes at room temperature.

4. Remove PBS and refill each well with 1 ml 1x PBS, allow washing on rotator for 10 minutes at room temperature.

5. Remove PBS and refill each well with 1ml 1x PBS **(exact volume)**, allow washing on rotator for 10 minutes at room temperature.

6. Remove 250µL of PBS and add 250µL methanol back to each well, allow washing on rotator for 15 minutes at room temperature *(wells now conatins750µL PBS: 250µL Methanol)*.

7. Remove 500µL PBS: methanol and add 500µL methanol to each well, allow washing on rotator for 15 minutes at room temperature *(wells now contains 500µL PBS: 500µL methanol)*.

8. Remove 750µL of PBS: methanol solution and add 750µL methanol to each well, allow washing on rotator for 15 minutes at room temperature *(wells now contains 250µL PBS: 750µL methanol)*.

9. Remove whole 1 ml of PBS: methanol solution and add 1 ml 100% methanol to each well, allow washing on rotator for 15 minutes at room temperature *(wells now contains 100% methanol)*.
   (**You may STOP and store for later; just seal with plastic wrap to avoid methanol evaporation, cover with aluminum foil and store in -20^{o}C freezer).**

10. Remove methanol and refill each well with 1 ml 1x PBS, allow washing on rotator for 15 minutes at room temperature.
11. Remove PBS and refill each well with 1 ml 1x PBS, allow washing on rotator for 15 minutes at room temperature.
12. Remove PBS and refill each well with 1 ml 1x PBS, allow washing on rotator for 15 minutes at room temperature.
13. **BLOCKING:** Block with 10% donkey serum in PBST for 1 hour at room temperature.
14. **PRIMARY ANTIBODIES:** Add primary antibodies (you could use multiple antibodies) (1:250 dilution in 1% donkey serum/PBST) incubate in 4°C refrigerator on rotator 1–2 nights *(2 nights sometime better)*.
15. Wash with PBST on rotator in **4°C** for 4–5 hours changing PBST **at least 5 times**.
16. **SECODARY ANTIBODIES:** Add secondary antibodies (1:1000 dilution in 1% donkey serum/PBST, incubate in 4°C refrigerator on rotator overnight *(24 hours or more)*.
17. Wash with PBST 4°C refrigerator on rotator overnight for 4–5 hours changing PBST **at least 5 times**.
18. Add nuclear stain (DAPI for 10–15 minutes) directly to secondary if needed solution; incubate for 30 minutes at room temperature.
19. Wash 6 times with PBST at 4°C each time incubate for 10 minutes on rotator.
20. Mount with Gelvatol mount media *(use dissection microscope to make sure lumen of intestine is facing up toward coverslip; tighten the coverslip and slide using small paper clips).*

Reagents:

- 4% PFA—Dilute one capsule of 16% PFA (Electron microscopy Sciences Cat # 15710) in 1x PBS.
- PBST—0.1% Triton-X 100 in 1xPBS.
- 10% donkey serum in PBST—add 100µL donkey serum in 900µL PBST.
- DAPI solution: DAPI (4',6-Diamidino-2-Phenylindole, Dilactate) (BioLegend cat # 422801)—To make 5 mg/ml (10.9 mM) solution, dissolve entire contents of vial in 2 ml of deionized water. Store between 2°C and 8°C, protected from light. For long-term storage, aliquot and store at -20°C.