Immunohistochemistry: pS6 (by Frank Ko)

Tissue Preparation:

1. Fix the bone samples in 4% PFA for 24 hrs at 4°C
2. Decalcify the samples with 20% EDTA at 4°C for 7 days (change the EDTA daily)
3. Paraffin processing (histocore)

Before starting:

- Make 1L 1X TBS
  - 100 mL 10X TBS
    - 10X TBS (1L): 60.5 g Tris (Fisher BP152)
      87.6g NaCl (Fisher BP358)
      pH to 7.5
      Bring to 1L w/ ~800 mL H₂O
  - 900 mL H₂O
- Prepare humid chambers (w/ Whatman paper)
- Make 4L 1X TNT
  - 400 mL 10x TBS
  - 3600 mL dH₂O
  - 2 mL Tween-20 (Sigma P2287): add while stirring

Staining process: (use TSA kit, Perkin Elmer #NEL700A)

1. Incubate slides at 60°C on slide warmer for at least 1 hr
2. Deparaffinize and dehydrate (use clean solutions!)
   a. Xylene, 3x 3 min
   b. 100% EtOH, 2x 2min
   c. 95% EtOH 2 min
3. Rehydrate in 1X PBS, 5 min RT
4. 1% H₂O₂/MeOH 20 min RT
5. Rinse quickly in 1X PBS
6. Circle sections with Pap-pen
7. Trypsin, 20 min 37°C in moist chamber
   a. Add 1 trypsin tablet (Sigma T7168-50) per 1 ml dH₂O
8. Rinse quickly with 1X TBS
9. Incubate with TNB at RT in moist chamber for 30 mins
10. 1° antibody diluted in TNB, RT in moist chamber for O/N at 4°C
    a. pS6 (Cell signaling #4858) 1:400 dilution in TNB
11. Wash: 1X TNT, 3x 5 mins at RT
12. Biotinylated 2° Ab diluted in TNB, in moist chamber for 30 min
    a. antiRabbit IgG, 1/800
13. Wash: 1X TNT, 3x 5 min at RT
14. SA-HRP (from kit) 1:100 dilution in TNB, 30 mins at RT

15. Wash: 1X TNT, 3x 5 min at RT

16. Biotinyl Tyramid (kit) 1:50 dilution in amplification diluent, 5 min RT

17. Wash: 1X TNT, 3x 5 min at RT

18. SA-HRP (from kit) 1:100 dilution in TNB, 30 min RT

19. Wash: 1X TNT, 3x 5 min at RT

20. For HRP detection, use DAB (VECTOR SK-4100)
   In 5ml dH2O:
   Add 2 drops buffer; mix well
   Add 4 drops DAB; mix well
   Add 2 drops H2O2; mix well
   Add 2 drops NiCl (optional)

21. Incubate until color develops (2-10 mins)

22. Wash 5 mins in dH2O

23. Counterstain (optional)

24. 95% EtOH 2 min

25. 100% EtOH 2x 3 min

26. Xylene 2x 5 min