Ploton Silver Stain (~3-3.5 hrs) **Alliston Lab**

Dewax: (in standard staining dish)

1. dewax slides 63° C on slide warmer or dryer for ~15 mins (Paraffin melted & transparent)
2. 100% Citra-Solve wash 5 mins (3X)

Rehydrate: (in standard staining dish)

3. 100% Ethanol wash 5 mins (2X)
4. 95% Ethanol wash 5 mins (2X)
5. 70% Ethanol wash 5 mins
6. ddH2O wash 5 mins (2X)

MAKE SILVER STAIN

Silver Stain: *(in disposable coplin jar)*

7. incubate in working silver solution in dark for 55 mins

Remove slides from disposable coplin jar.
Rest of instructions are done in standard staining dish.

8. Rinse in H2O 5 mins (3X)

MAKE SODIUM THIOSULFATE

9. Incubate in 5% sodium thiosulfate 10 mins
10. Rinse in H2O 5 mins (3X)

Cresyl Violet: (*Nuclei Stain- Optional*) (in standard staining dish)

11. 0.1% cresyl violet working solution for 3-10 mins
12. Rinse in tap water for 1 min
13. Wash in 70% EtOH for 3-5 dips

*proceed with steps 14-16 with ~5-10 dips instead of 5 mins

Dehydrate: (in standard staining dish)

14. 95% Ethanol wash 5 mins
15. 100% Ethanol wash 5 mins (2X)
16. 100% Isopropyl alcohol- 5 mins
17. 100% Citrasolve- 2-5 mins (3X) (no streaks!)

Coverslip:

18. Use mounting media (Coversafe) to coverslip

Gelatin-formic acid (10ml):
10ml warm water (50-55° C) *(can microwave for ~15 secs)*
0.2g gelatin type B, 75 bloom (G-6650) or type B, 100 bloom (G8500)
*vortex to dissolve

after gelatin dissolves & cools to room temperature
add 125ul 90% formic acid

50% Silver Nitrate (15ml):
15ml water
7.5g silver nitrate

*very unstable- add H2O right before use
*shake gently to mix & keep in dark
(DO NOT VORTEX)
(make fresh-solution should be clear prior to use)

Working Silver Nitrate Solution (20ml)
15ml 50% silver nitrate
7.5ml gelatin-formic acid
*shake gently to mix & keep in dark

5% Sodium Thiosulfate (250ml):
250ml water
12.5g sodium thiosulfate
Pentahydrate (S445-500)

*vortex to dissolve
(good for large staining jar ~250ml)

Hazardous waste:
-dispose working silver stain, gelatin-formic acid & 1st water rinse in appropriate hazardous waste according to institute's EH&S

Tips:
*other permutations of volumes, incubations, etc. have been tested but this protocol provides the best consistent results on mouse and human specimens
*stains everything-use bench diaper & change gloves often
*make all reagents **fresh** (batch is good for 1 disposable coplin jars)
*can do multiple batches at a time

*use **disposable coplin jar** for step 7 (Evergreen Scientific 222-5450-G8S) (holds 20-25ml; max 4 slides)

-change gloves often
-do NOT use/work with metal instruments
-use paraffin coated forceps tips to pick up slides

-dirty glassware will cause precipitation of silver on slides (often seen using dropwise method)
    -use clean water bath
    -dry slides in clean racks (NOT slide dryer)
    - wear gloves when sectioning & handling slides
    -dishes should be clean (NOT acid cleaned containers)

-can work on frozen sections (tested on 02/27/18)
    -must mount using Aqueous mounting media (skip Dehydrate Steps)
    (updated 10/02/2020)
    -decal bones fall off of slides; use adhesive slides and try longer drying (O/N)
    -can possibly get sections using cryojane (stains aren't as good as on paraffin)

Clean Up Silver Nitrate:
-clean stained dishes in concentrated nitric acid (1min)
-work in fume hood & wear PPE

References:
Dole, N.S., Mazur, C.M., Acevedo, C., Lopez, J.P., Monteiro D.A., Fowler, T.W., Gludovatz B., Walsh F., Regan, J.N., Messina, S., Evans, D.S., Lang T.F., Zhang B., Ritchie R.O., Mohammad, K.S., and Alliston, T. (2017). Osteocyte-Intrinsic TGF-beta Signaling Regulates Bone Quality through Perilacunar/Canalicular Remodeling. Cell Reports 21, 2685-2596.

Fowler, T.W., Acevedo, C., Mazur, C.M., Hall-Glenn, F., Fields, A.J., Bale, H.A., Ritchie, R.O., Lotz, J.C., Vail, T.P., and Alliston, T. (2017). Glucocorticoid suppression of osteocyte perilacunar remodeling is associated with subchondral bone degeneration in osteonecrosis. Sci. Rep. 7, 44618

Ja´ uregui, E.J., Akil, O., Acevedo, C., Hall-Glenn, F., Tsai, B.S., Bale, H.A., Liebenberg, E., Humphrey, M.B., Ritchie, R.O., Lustig, L.R., and Alliston, T. (2016). Parallel mechanisms suppress cochlear bone remodeling to protect hearing. Bone 89, 7–15.

Cresyl Violet (Nissl Stain):

0.1% Cresyl violet solution (100ml): (pH 3.56-3.6)

Cresyl violet acetate or cresyl echt violet	0.1 g (or 100mg)
Distilled water	100ml
Glacial Acetic Acid	300ul (just before use)

*mix for at least 30 mins
*filter to remove precipitated particles (optional)
*keep in dark

Directions:

1. 0.1% cresyl violet working solution for 3-10 mins (did 10 mins)
2. Rinse in tap water for 1min
3. Wash in 70% EtOH for 3-5 dips (did 3 dips)
4. Differentiation solution for 2 mins (optional-did not do)

*results: nuclei is dark blue, very nice contrast
Also stains cartilage and some un-mineralized bone
*for thicker sections (20-50um), stain in warm solution (37-50C) can improve penetration and enhance even staining

Reference:
http://www.ihcworld.com/_protocols/special_stains/nissl.htm

Notes:
-This stain works well with more distinct nuclei stain and less staining of unmineralized tissue compared to cresyl violet recipe w/ sodium acetate
-works well with both SN that has gelatin type A bloom 275 and gelatin type B 100 bloom (gelatin type B has less background in SN stain)