**Axon-Myelin stain protocol**

**Day 1:**

1.Bring 1 litre of Sodium Citrate buffer to a boil for step 4.

2. **Warming slides**, put sections on slide warmer at 60°C for 30 min.

3. **Deparaffinizing and rehydration**, sequentially immerse warmed slides in:

 Xylenes **→** 15 min @RT

 1:1 of xylenes and ethanol **→** 10 min @RT

 100% ethanol

 95% ethanol

 75% ethanol 5 min @RT

 50% ethanol

 Millipore H2O

4. **Antigen retrieval**, in sodium citrate buffer, in pressure cooker for 45 min and then cool to RT. Wash 3 times with

 1x PBS, 5 min each. Water rinse.

5. **Quenching endogenous peroxidase,** incubate slides in 3% H2O2 in PBS at RT for 5 min. Wash three times with 1x PBS, 5 min each.

6. **Blocking**, incubate slides, in a humidified chamber, with 5% BSA plus 0.3% Triton-X 100 in PBS for 30 min at RT.

7. **Primary antibody**, incubate blocked slides, in a humidified chamber, with a primary antibody SMI-31 at 1:50,000 titer at

40C overnight. 1**°** Ab diluted in blocking solution.

**Sodium Citrate Buffer** (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0):

 Tri-sodium citrate (dihydrate) 2.94 g

 Tween 20 0.5 ml

 Distilled water **↑**1000 ml

**Day 2:**

1. Next day, **wash** 3 times with 1x PBS, 5 min each.

2a. **Secondary antibody,** incubate in 1:200 biotin-conjugated secondary antibody (from Vectastain Elite ABC Kit) in PBS or blocking solution at RT for 1h. Wash 3 times with 1x PBS,

 5 min each.

2b. Make **ABC complex** during 2° incubation and keep on ice.

 → 2 drops each A & B to 5 ml PBS

 When PBS washes start, move ABC complex to RT.

3. **ABC** for 30 minutes.

3. **Develop colour with DAB**: 4.5 ml PBS

 0.5 ml 5 mg/ml DAB

 1.7 μl H2O2 → added just prior to use

 DAB stock = 5 mg/ml in PBS (make in bulk and freeze)

 *N.B.* for optimal density and low background, watch colour change by eye (about 30 seconds) and also under microscope.

4. **Dehydration** in the reverse sequence to rehydration, dehydrate

until in 95% EtOH.

 5. While dehydrating, pre-set incubator to 56ºC.

 6. Incubate in 1% Luxol fast blue for **48 hours @**56ºC.

**Day 4:**

1. Rinse in distilled water

2. Differentiate in 0.05% Lithium Carbonate for 30 seconds.

3. Differentiate in 70% EtOH for 30 seconds (watch carefully and use

less time if stain is coming off quickly)

4. Look under microscope to check if grey matter and white matter

are clearly defined and if myelin is stained sufficiently to differentiate from axonal stain.

5. Rinse in distilled water for 30 seconds (note: do not use 70%

EtOH as stain will come off).

6. Complete dehydration starting in 100% EtOH for 5 min, then 1:1

Xylenes+EtOH, and finish dehydration with Xylenes for 5 min.

 7. Mount with permount and coverslip.