*04/18/20016 written by Mooney*

**Whole mount staining; embryonic lung protocol**

1. Fix the tissue (that should be smaller than E13.5) with 4% PFA (dissolve with PBS or TBS) overnight
2. Wash with PBS x3
3. Use Blocking reagent (M.O.M kit from Vector) for 2hrs. if you will use mouse primary antibodies. If not, block with 5% Horse serum or 0.75% BSA for 4hrs. in staining buffer: 0.03% Triton X + PBS
4. Incubate with primary antibody in the staining buffer (0.03% Triton X + PBS) overnight in cold room

Ex. Sox2, Sox9, Nkx2-1; each 1/100 dilution. in buffer (Primary antibodies with 0.03% Triton-X, 0.75% BSA in PBS)

1. Wash with PBS+ 0.03% Triton-X for 1hr.
2. 2ndary antibodies for 4hrs with 0.03% Triton-X, 0.75% BSA in PBS
3. Prepare clearance reagent

*Buffer S*; 10mM Tris-HCl pH 7.4 + 0.267mMEDTA + 2% Saponin (1M Tris-HCl pH 7.4 stock 100ul, Saponin 0.2g, dH2O 10ml, 5M EDTA 4ul)

*Dilute Omnipaque in buffer S by following order*

**Reagent A.** 1/3x Omnipaque or Histodenz (Sigma Cat#. D2158) 1.12g /10ml Buffer S

**Reagent B.** 1/2x Omnipaque or Histodenz (Sigma Cat#. D2158) 1.75g /10ml Buffer S

**Reagent C.** 1x Omnipaque or Histodenz (Sigma Cat#. D2158) 3.5g /10ml Buffer S

**Reagent D.** 1x Omnipaque or Histodenz (Sigma Cat#. D2158) 7.55g /10ml Buffer S

1. Incubate samples with Reagent A for 6-10hrs. at R.T.
2. Switch to Reagent B and incubate for 6-10hrs. at R.T.
3. Switch to Reagent C and incubate for 12hrs. at R.T.
4. Switch to Reagent D (SeeDB2S) and incubate for 12hrs. at R.T.

You might be able to change Triton X to Saponin based Buffer S during staining, but it could depend on antigen. In the high-resolution imaging with SeeDB2S, the WD (**typically 200-300 um**) is the limiting factor for depth. Saponin stock solution should be sterilized by filtration. Sodium azide should be added for longer storage.

SeeDB2S samples should be stored at room temperature.

Previously, 97% TDE has been proposed for this purpose (Staudt et al., 2007), but some of the common fluorescent proteins and dyes were quenched in TDE. SeeDB2 preserves many of the fluorescent proteins and dyes (Alexa). So, use SeeDB2 samples ASAP if you performed immunostaining.

<https://sites.google.com/site/seedbresources/seedb2-protocol>