**Basic Immunofluorescent Staining Protocol
for Free Floating Sections**

**Day 1: NOTE: fixed tissue o/n (drop fix). Leave in 30% sucrose @ 4C until tissue sinks. This step is tissue type dependent and may vary.**

1. **Embed tissue in gelatin. Freeze on dry ice until completely solidified. Ice down stage of sliding microtome with dry ice.**
2. **Section tissue at \_\_\_µM, collect sections in tissue catcher filled with 0.1M PB**
3. **Melt gelatin off of sections by capping tissue catcher & running under trickling hot h20**
4. **Rinse with 0.1M PB and then run again under hot H20 to be sure that all gelatin is removed.**
5. **Rinse and agitate tissue gently on shaker laid horizontally within the tissue catcher 5X5 minutes with PB. Be careful to rinse lid each time with tissue catcher so that no tissue is lost during the washes.**
6. **Meanwhile prepare blocking solution** 5% normal sera (of the species that the 2ndary antibody is produced in (NDS for DAG):0.1M PB:1.0% Triton X.

 **Blocking Solution (add sera from species of interest to the following mixture)**250ul *donkey* sera *(because using donkey 2ndaries, see below)*
1 ml PB
40 µl 25% Triton X Solution

\*Note TX is very viscous, best to make a 10-25% stock and use from that rather than pipetting from the bottle each time

1. **Remove PB and add blocking solution. Seal lid with parafilm and agitate 1h to o/n @ RT.**

**\*\*NOTE MAY STOP HERE FOR THE DAY and CONTINUE on DAY 2 or STOP after adding Primary, step 9 below\*\***

1. **Prepare primary antibody in 0.1M PB 1ml/well depending on well size**

**Ab name/#: Rabbit Antibody Y
Lot#**

**Dilution used: 1:200, 5ul into 1ml 0.1M PB**

**Ab name/#: Goat Antibody X
Lot#**

**Dilution used: 1:500, 2ul into 1ml 0.1M PB**

1. **Add primary antibody. Agitate gently at RT *IN DARK* for 2-3h or o/n depending on manufacturers guidelines and previous testing results.**
2. **Wash 5X5 minutes w 0.1M PB. Agitate gently during washes.**
3. **Add secondary antibody-typically 1:500 to 1:1000 of Alexa Fluor 2ndary. Depends on species of primary.**

**Ab name/#: 488 *Donkey* Anti Goat
Lot#**

**Dilution used: 1:500, 2ul into 1ml 0.1M PB**

**Ab name/#: 568 *Donkey* anti Rabbit
Lot#**

**Dilution used: 1:1000, 1ul into 1ml 0.1M PB**

1. **Allow 2ndary antibody to bind for 2-3h at RT or o/n IN DARK. Gently agitate.**
2. **Wash 5x5 with 0.1M PB in dark. Agitate gently during washes.**
3. **Wash 1-2X with diH20 to remove salts from PB.**
4. **Prepare slide for mounting sections. Add small drops of H20 to + (Fisher) or subbed slide.**
5. **Remove sections from tissue catcher to dissection dish. Using low light on the dissecting scope, place slices onto previously prepared slide. Check tissue under scope for orientation and dry remaining of H20 drops using kim wipes.**
6. **Place slide in dark to air dry. Add slo fade or prolong gold mounting media and gently coverslip trying to avoid air bubbles. Dry at RT and SEAL with nail polish the next day for ProLong or immediately for Slo Fade.**
7. **Allow to harden o/n and view the following day on fluorescent scope.**