**Kordower Lab: Immunofluorescence Multi-label protocol (Same-species)**

**DAY 1 (**4 hrs**):**

1. Wash sections (6 x 10 min) in DM.
2. Endogenous peroxidase inhibition (20 min). 0.1M Sodium meta-periodate in TBS. (only need if using ABC b/c it has HRP and could cause background).
	1. 100 mL TBS + 2.13 g sodium meta-periodate
3. Wash (2 x 10 min) in DM (only if you did step 2).
4. Serum blocking step (1 hour incubation):
	1. 100 mL DM
	2. 3 mL normal serum
	3. 2 g BSA
5. Incubation in primary antibody (18 - 72 hrs) \*\*Optionally, refrigerate to keep antibody stable\*\*
	1. 100 mL DM
	2. 1 mL normal serum
	3. 1 g bovine serum albumin (BSA)
	4. 0.5 mL triton X100

**DAY 2 (**8 hrs**):**

1. Wash (6 x 10 min) in DM
2. Fluorophore- conjugated secondary antibody incubation (1 hour) Concentration of secondary antibody is 1:200
3. 100 mL DM
4. 1 mL normal serum
5. 1 g BSA
6. Wash (2 x 10 min) in TBS
7. Serum blocking step (1 hour incubation)
8. 100 mL TBS
9. **5% (v/v) (1:20 dilution)** normal serum (50 mg/mL) (**FROM PRIMARY ANTIBODY HOST**) \*saturates open binding sites on secondary antibody with mouse IgG
10. 1 g bovine serum albumin (BSA)
11. Oversaturate with Fab antibody **against host of primary antibody** and from same host species as secondary antibody. (ex. If primary was mouse and secondary was goat anti-mouse, you would use a Fab-goat **anti-mouse** antibody). Working concentration: 40 µg/ml. (1 hour incubation)
	1. 100 mL TBS
	2. 40 µg/ml Fab antibody (base concentration is 1.3mg/mL for fab-goat anti mouse, so use M1V1 = M2V2 to find V1/”x”).
12. Wash (2 x 10 min) TBS
13. Incubation in **2nd primary** antibody (18 - 72 hrs) \*\*Optionally, refrigerate to keep antibody stable\*\* **ALSO ADD 3RD PRIMARY ANTIBODY FROM DIFFERENT SPECIES IF NEEDED**. (I.e. rabbit primary)
14. 100 mL TBS
15. 1 mL normal serum
16. 1 g bovine serum albumin (BSA)

**DAY 3:** (2 hrs)

1. Wash (6 x 10 min) in TBS

2. Fluorophore- conjugated secondary antibody incubation (1 hour) Concentration of secondary antibody is 1:200 (**SAME AS DAY 2 STEP 2, BUT DIFFERENT FLUOROPHORE**. Ex. goat anti-mouse and goat anti-rabbit with different colors.) **ADD FLUOROPHORE FOR 3RD PRIMARY ANTIBODY AS WELL!**

a. 100 mL TBS

b. 1 mL normal serum

c. 1 g BSA

3. Wash in TBS (3 x 10 mins) \*Can add DAPI (1:15,000) during the second TBS washing step.

4. Can leave in TBS in the refrigerator.

**Control for Fragment antibody (Fab):** Control tissue should be processed alongside experimental tissue through Day 2 (step 6). Skip 2nd primary incubation all together (step 7), and complete Day 3. Check under microscope to ensure there is no co-labeling between the two chosen fluorophores**.**

**Use appropriate +/- controls.**

**DO NOT USE** any detergent (i.e. triton x-100, tween-20, DM) from step 3, onward. Detergent will wash away the fragment antibodies.