IMMUNO EM PROCEDURES

Pre-embedding of tissue:

FIXATIVE:
4% PFA + either 0.1%, 0.25%, 0.5% or 1% gluteraldhyde.

- PFA should always be fresh
- Glut should always be used fresh from sealed container
- Higher glut, less antigenecity, better ultrastructure

1. Perfuse fixative in mouse through left ventricle of the heart for 5 minutes. Quickly remove tissue of interest with continual drip of fixation over the tissue while it is being dissected out. Place tissue in fixation for one hour at 4˚C.

- Samples should immediately be moved to steps below

the following steps are conducted at 4˚C unless otherwise noted – on rotater or rocker

2. Samples washed in 3 x 10 min changes of filtered dist water
3. Dehydration:

40%	ETOH	10 min
60%	"	"
80%	"	"
100%	"	2 x 10 min.

following the second 100% ETOH, samples are equilibrated to room temperature, and subsequent steps carried out at RT unless otherwise noted

4. Infiltration: (Note: LRW = L.R. White, London Resin Company) – rotating or rocking

2:1	100% ETOH:LRW	1 hour
1:1	100%ETOH/LRW	2-3 hrs (or overnight)*
1:2	100% ETOH/LRW	2 hrs (or overnight)*
1:4	100% ETOH/LRW	2 hrs (or overnight)*
	100% LRW I	1-2 hrs. (or overnight)*
	100% LRW II	2-3 hrs.
	100% LRW III	2-3 hrs.

Embed in **gelatin** capsules in fresh LRW

5. Polymerize for 24 hours at 45˚C. (Note: do not polymerize at the usual 60˚C...too hard on antigens)

6. Ultrathin sectioning: collect sections (75-80nm in thickness) on nickel grids (important for immuno

Post-embedding immunostaining:

1. Hydrate grids in buffer (TBS)10 min at RT
2. Treat with 0.01M glycine in buffer for 10 min (deactivates reactive aldehydes) at RT
3. Block with "blocking solution" (either for goat or rabbit antibodies (purchased from Aurion or using Isakson Lab blocking solution) for an hour at RT
4. Add primary antibody in blocking solution at 4˚C overnight in a moist chamber (for initial studies, this likely requires logrhythmic concentrations, e.g., 1:50 and 1:500)
5. Wash in 3 x 5 min in blocking solution at RT
6. Secondary antibody: appropriate dilution of secondary antibody (e.g., 1.5X primary concentration, so 1:50 primary would be 1:75 for secondary; this is good as a start and can dilute as needed) conugated to colloidal gold (gold size depends on experiment; we use 10 nm gold beads from jackson) in blocking solution for 2 hrs RT
7. Wash 3 x 5 min in blocking solution at RT
8. Wash in 3 x 5 min filtered dist water at RT
9. Air dry (approximately 2 hours RT)
10. Contrast stain with lead citrate and uranyl acetate
11. carbon coat for viewing under TEM