Zebrafish Electron Microscopy Fixation

1. Prepare fixative (1 ml for larvae and 20 ml for adult) without glutaraldehyde
   - 200 ul 20% EM grade PFA (4 ml)
   - 500 ul 0.2M CaCo (10 ml)
   - 200 ul ddH2O (4 ml)
   - 4 mg CaCl2 (80 mg)
2. Anesthetize larvae or adults in tricaine and transfer larvae into a microcentrifuge tube (less than 40 embryos/tube) or a 50 ml falcon tube for adults
   - For earlier stages, make sure they are dechorionated
   - Remove as much media as possible
3. Add the 900 ul of fix to the tube and wait for fish to be completely dead (ie, no more twitching) rocking at RT
   - For adult brains, I remove the dorsal skull to expose the neural tissue directly to the fixative but leave the brain sitting in the fish
4. Add 100 ul of 50% glutaraldehyde to the fixed fish (2 ml for adult). Leave tubes rocking in secondary container at RT for days until they begin to yellow
5. Wash in 0.1M CaCo with a few washes at RT and then O/N at 4C
6. Entire larval heads or 50 um thick vibratome sections of adult brains were submitted to the EM facility for post-fixation (1% osmium tetroxide and 1.5% potassium ferrocyanide), dehydration, embedding in expoxy resin, and ultrathin sectioning (80 nm). If not doing tracer leakage assays, sections were counterstained with Reynold's lead citrate prior to imaging

Tracer Leakage Assays: 2.3 nl of 5 nm NHS-activated gold nanoparticles (Cytodiagnostics: CGN5K-5–1, ~1.1^{14} particles/ml in PBS) were injected into the cardiac sac just as for the fluorescently conjugated tracers. After 5 min of circulation, the fish were fixed for electron microscopy as above.