

### Neomycin hair cell toxicity assay

Raise zebrafish embryos at 28.5°C in 100 mm petri dishes containing embryo medium.

Feed newly hatched free-swimming larvae paramecium and dry fish food at 4 days post fertilization with lights on. We find feeding larvae improves the reproducibility of all assays.

For treatment, transfer fish to baskets and place within a well of a 6-well plate (e.g. BD Falcon flat bottom plates, Fisher cat. 08-772-49; BD Biosciences No.:351146) containing 6 milliliters of 1x embryo medium. Typically, tests are done with ten fish per basket but work well with up to 50 fish. All treatment and wash volumes are 6 mL. Baskets are 50 ml Falcon tubes with hole cut in lid and mesh glued in place. Alternatively, we use tissue culture inserts: Corning Netwell, 6-well cluster, 24 mm dia, 74 um mesh, Fisher catalog number 07-200-213 (Corning 3479).

Treat with neomycin for 30 minutes.

Neomycin is diluted in embryo medium to concentration of 0 to 500  $\mu$ M (neomycin sulfate, Sigma, St. Louis, MO, catalog # N1142).

Rinse fish briefly 3 times in embryo medium and allow recovery for 45 minutes.

Add 700  $\mu$ l of .05% DASPEI (2-{4-(dimethylamino)styryl}-N-ethylpyridinium iodide, Invitrogen Molecular Probes, cat# D426) and stain for 15 minutes (i.e. total post neomycin recovery time of 1 hour).

Rinse twice in embryo medium.

Add MS-222 (3-aminobenzoic acid ethyl ester, methanesulfoneate salt, Sigma, cat. # A5040) to anesthetize. Typically each well in a 6-well plate holds 6-8 ml. Add 350  $\mu$ l of 0.4% stock, pH 7.2.

View with epifluorescence dissecting microscope equipped with a DASPEI filter set (excitation 450-490 nM and barrier 515 nM, Chroma Technologies, Brattleboro, VT). Will work with other filters but the DASPEI filter set improves imaging.

For assessment of initial dose response curves, DASPEI staining of ten neuromasts (SO1, SO2, IO1, IO2, IO3, IO4, M2, MI1, MI2 and O2) on one side of an animal are evaluated. Each neuromast is scored for presence of DASPEI staining (score=2), reduced DASPEI staining (score=1) or absence of DASPEI staining (score=0). Total scores for an animal are tabulated, to give a composite score that can range from 0 to 20. Average scores and standard deviations are calculated for animals in each treatment group.

1x embryo media (standard lab EM):

1 mM MgSO<sub>4</sub>,  
0.15 mM KH<sub>2</sub>PO<sub>4</sub>,  
0.05 mM Na<sub>2</sub>HPO<sub>4</sub>,  
1 mM CaCl<sub>2</sub>,  
0.5mM KCl  
15 mM NaCl  
0.7 mM NaHCO<sub>3</sub>.

1x embryo medium is made fresh weekly. A 20x stock of embryo medium is prepared by adding components in the order listed (to prevent precipitation of calcium phosphate), omitting the sodium

bicarbonate. Sodium bicarbonate is made as 500x stock at pH 7.2 and added during preparation of 1x embryo medium. Check pH of 1x stock.

#### FM1-43 as a mechanotransduction marker

Hair cells can be visualized with FM1-43 (or the fixable analog FM 1-43FX; Invitrogen cat. # T-35356 and F-35355 respectively). Rehydrate dye in distilled water to 1mM. FM1-43 works well at 1-3  $\mu$ M in embryo media.

Immerse fish in baskets in 3 micromolar FM1-43 FX for 30 seconds. Short incubation times are critical since FM1-43 can also be internalized by endocytosis.

Rinse immediately in 1x embryo media four times.

Hold 15-20 minutes prior to aminoglycoside treatment (as described above).

If fixing, euthanize fish in ice cold embryo medium with MS222 and transfer to eppendorf tubes.

Fix in cold 4% PFA in PBS, 1 hour at RT.

Replace with fresh 4% PFA and fix overnight at 4°C.

Rinse 3 x in 1x PBS.

Rinse in 50% glycerol: 50% PBS and view.

#### DNA dyes as hair cell nuclear markers

Hair cells can be visualized with the DNA dyes To-Pro-3 and Yo-Pro-1 (Invitrogen T3605 and Y3603) as described (Santos et al., 2006). To-Pro-3 has an excitation/emission spectrum with maxima at 642/661; Yo-Pro-1 has maxima at 491/509. Either can be used in conjunction with FM 1-43 staining. However, the dyes do not fix well.

Immerse fish for 1 hour in 2  $\mu$ M nuclear dye in EM, followed by 3x rinsing in EM.