PROTOCOL FOR FIXING AND STAINING JUVENILE LIGHT ORGANS FOR VISUALIZATION OF CRYPT SPACES AND/OR CRYPT EPITHELIA

[Transcribed From L. Sycuro (11/2002) by M.S. Wollenberg (05/2009)]

Fluorescent Probes

- Rhodamine phalloidin [Molecular Probes (Invitrogen)]

Stains cytoskeletal F-actin. Illuminates the epithelial brush border nicely, outlining the crypt lumen.

Stock = add 1.5mL MeOH to vial contents (1 mg). Protect from light; store at -20C.

Working solution = pipette 5uL into a dry ufuge tube; allow to dry (~20min). Reconstitute in 200uL mPBS + 0.5% TritonX and use immediately.

- BODIPY-FL paclitaxel [Molecular Probes (Invitrogen)]

Stains tubulin in cytoplasm.

Stock = add 125uL DMSO to vial contents (10ug); pipette 25uL aliquots. Protect from light; store at -20C.

Working solution = add 75uL of mPBS + 0.5% TritonX to a 25uL of stain. Working solution can be reused a maximum of 3 times.

Solutions Required

- mPBS (marine phosphate-buffered saline)

Make 1M monosodium phosphate monohydrate and disodium phosphate heptahydrate stock solutions from monobasic sodium phosphate and dibasic sodium phosphate, respectively. Make 5M sodium chloride stock solution.

Prepare 1X mPBS, pH 7.4 (50mM NaH2PO4-buffer strength, 0.5M NaCl)
213.0 mL autoclaved DI H2O
25.0 mL 5M NaCl
10.4 mL 1M disodium phosphate heptahydrate (Na2H)
1.6 mL 1M monosodium phosphate monohydrate (NaH2)
check pH is 7.4 with electrode
Store at 4C.

- 4% formaldehyde

Make mPBS with 0.45M NaCl (see above, except add 22.5mL 5M NaCl and 215.5 mL DI H2O). [Only use this stock for formaldehyde, for everything else use mPBS w/ 0.5 NaCl]

Add 5.4mL 37% formaldehyde to 44.6mL 1X mPBS (0.45M NaCl) to make 4% working solution.

Store at 4C.

- Mounting Medium (VectaShield may be substituted)

Add 95mL clear glycerol to 5mL mPBS containing 5mg paraphenylenediamine (ppd helps minimize bleaching, make sure it is in solution before adding to glycerol).

Protect from light. Stir until no phase differences are apparent (long time).

Keep at 4C in a foil-covered bottle. Solution will gradually discolor; discard when it turns orange or bleaching becomes a problem (with regular use - after 4-6 weeks).

- 1% TritonX-100 and 0.5% TritonX-100 in 1X 0.5M NaCl mPBS

Make 10mL of 10% TritonX-100 and dilute 1:10 and 1:20 respectively.

Store at RT for no more than a few weeks.

Procedure
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1. Anesthetize squid at desired time point with seawater containing 2% EtOH.

2. Pith animal(s) and dissect open mantle and funnel.

3. Fix animal(s) with 4% formaldehyde for 1 hour at room temperature.

4. Transfer squid to mPBS.

4a. (Squid may be stored up to 3 weeks at 4C at this step.)

5. Dissect out light organ and transfer animal(s) to 0.5mL tube containing fresh mPBS.

6. Carry out this and subsequent steps with tubes on rotator at RT. Wash LOs 2X in mPBS, 10 min. each wash. Remove mPBS using 1 or 3 cc syringe with small gauge needle. Go slowly; keep beveled opening of needle against side of tube to avoid accidentally sucking up LOs.

7. Permeabilize LOs using 1% TritonX-100/mPBS for 20 minutes at RT.

8. Incubate LOs in paclitaxel working solution for 20 minutes at RT. Protect from light and keep tubes wrapped in foil from this point on.

9. Wash LOs 2X in mPBS, 10 min. each wash. While washing, in a separate receptacle, aliquot 5 uL of rhodamine phalloidin to allow time for the methanol to evaporate.

10. Incubate LOs in phalloidin working solution for 20 minutes.

11. Wash LOs 2X in mPBS, 10 min. each wash.

11a. (LOs may be stored in mPBS at 4C for up to 3 weeks at this step, before mounting as below.)

12. Paint 1 or 2 square boxes on glass slides with nail polish, dry.

13. Mount LOs by transferring each one, individually, to the center of a drop of glycerol mounting medium inside a given nail polish box. Make sure excess mPBS is removed from each LO before transfer to slide - best done by blotting LO against tube rim several times. After LOs are in glycerol, orient with a

dissecting scope so that pores and LO are facing 'up' or away from slide base. Cover LOs/glycerol with coverslip and paint edges with nail polish to prevent slide drying.

14. View with confocal or epifluorescent microscope.

15. For any given experimental design, steps 8, 9, and/or 10 may be left out, as necessary.

16. (MSWollenberg; 2009.05)
Personally, I have found no difference in fully dissecting LOs from animals at step 2, followed by immediate fixation, instead of partially dissecting and fixing as listed above.

MORE important, in my opinion, is that an attempt is made to puncture the ink sac, which sits above the majority of crypt 1. Ink in the ink sac may obscure microscopic visualization of this area, using a very fine pair of tweezers or very narrow gauge needle should do the trick. Also important is that the researcher is careful not to rip apart the crypts/LO when attempting to puncture the ink sac. It may be easier to puncture the ink sac after fixation, although, anecdotally, I prefer to do it before because the tissue is more delicate and easily ruptured...

A good compromise is to fix the squid, whole and alive, for a minute or so in 4% formaldehyde before dissecting the LO and puncturing the ink sac, this makes the tissue 'spongy' and more easily dissectable, while also effectively anesthetizing/immobilizing the animal.