Immunostaining to label central canal cilia

This protocol was optimized by Elizabeth Bearce (last updated: 07/21/20) from an initial protocol for staining younger embryos by Jose Pelliccia.

Objective:

This protocol describes how to perform immunostaining of acetylated alpha-tubulin (cilia, neurons) and gamma-tubulin (microtubule organizing centers) in early larvae, with optional steps for Hoechst (nuclei) and Phalloidin (actin - cell borders & musculature) co-stains.

It has been validated for embryos 24 – 48 hpf; it may work beyond these stages with modifications. Earlier embryos will be more fragile, and require less severe permeabilization. Later stages will require more aggressive permeabilization, and modifications to dissection & mounting protocols. Your mileage may vary.

Equipment:

1.5mL Eppendorf Tubes
Disposable 7mL plastic pasteur pipettes
Nutator (4C and RT)
“Dark Box” or Aluminum Foil
Standard Glass Slides
Confocal Grade Coverglass (#1.5)
Aquamount mounting medium
Forceps, eyelash tool
Electrical Tape
Nail Polish

Immunostaining Reagents
Mouse anti-acetylated alpha-tubulin (Sigma T6793)
Rabbit anti-gamma tubulin (Sigma T5192)
Goat Anti-Mouse conjugated with Alexa Fluor 488 (ThermoFisher A-11001)
Goat Anti-Rabbit conjugated with Alexa Fluor 546 (ThermoFisher A-11035)
Alexa Fluor 647 Phalloidin (ThermoFisher A22287)
Hoechst 33528 (Sigma 94403)

Note: When replacing solutions, tip Eppendorf tube upright and allow embryos to collect in the bottom. Remove as much liquid as possible, using a plastic pipette or vacuum aspiration. Pipette new solution gently down one side of the tube, then place tube immediately on its side to keep embryos from clumping. Rock as required, proceed to next solution.

Protocol:

1. Dechorionate and sort embryos. Euthanize with an overdose of Tricaine.

2. *Fixation*. Transfer 10-50 fish to 4% PFA (recipe below), which is pre-aliquoted into individual use tubes. (Do not exceed 50 per tube, and keep numbers equivalent between conditions/tubes.) Rock with gentle agitation overnight at 4°C. Do not exceed 18 hours.

3. A secondary methanol fixation at this stage can improve tubulin labeling, and allows for embryo storage for later staining. However, as phalloidin affinity is destroyed by methanol, the following steps will vary depending on desired co-staining protocols.

A. <u>If Phalloidin co-labeling is not required</u>:

i. *Permeabilization and secondary fixation*. In fume hood, remove PFA solution from embryos, and replace with 100% methanol. Perform 2-3 rinses in MeOH, to ensure removal of PFA solution. Discard all PFA and MeOH waste appropriately. Embryos can then be stored in MeOH at -20°C for future staining. If proceeding to staining steps immediately, rock embryos for at least 2 hours in MeOH at room temp, and then continue to rehydration steps.

ii. *Rehydration*. Remove methanol solution. Perform gradual rehydration by performing 5 minute rinses in each of the following: 75% Methanol in PBSTween, 50% Methanol in PBSTween, 25% Methanol in PBSTween, and PBSTween.

iii. *Rinse*. Perform three more 5 minute rinses in PBSTween. Proceed to blocking steps.

B. <u>If co-labeling with Phalloidin</u>, perform an alternative permeabilization. This step uses a stronger detergent than the rest of the protocol.

i. Remove PFA solution in fume hood, and perform three brief rinses into PBS, to remove any remnant PFA. Dispose of all PFA waste appropriately.

ii. *Permeabilization*. Remove PBS, and replace with PBST (recipe below). Rock for 30 minutes. Repeat 2 more times, for a total of 1.5 hours in 3 changes of PBST. Proceed with blocking steps.

4. *Block*. Remove PBST (or PBSTween), and replace with Blocking Solution (recipe below). Block for 2 hours, rotating at RT.

5. *Apply primary antibodies*. Make primary antibody cocktail in Primary Antibody Diluent (recipe below). Remove excess blocking solution, replace with primary cocktail. Rock gently at 4°C overnight.

   Note: These antibodies can be reused and saved, but I typically have not done so. If you're reusing antibody solution, do not make the cocktails in antibody diluent— perform the incubation in regular PBS.

- 1:600 rabbit anti-gamma-tubulin
- 1:500 mouse anti-acetylated alpha-tubulin
- Make 0.5 mLs of antibody solution per tube of embryos

6. *Rinse*. Remove antibody solution, and replace with Rinse Solution (recipe below). Rock for 30 minutes at RT. Replace the rinse solution with fresh rinse. Repeat 5 times total (2.5 hours).

7. *Apply secondaries*. Make secondary cocktails in rinse solution. If using Hoechst and Phalloidin, these will also be included here. Replace rinse solution with secondary cocktail, move embryos to a Dark Box (or wrap tubes in foil) and rock gently at 4 °C overnight.

- 1:500 goat anti-mouse conjugated with Alexa Fluor 488
- 1:500 goat anti-rabbit conjugated with Alexa Fluor 546
- 1:1000 Hoechst

- 1:100 Alexa Fluor 647 conjugated Phalloidin

8. *Rinse*. Remove antibody solution, and replace with Rinse Solution (recipe below). Rock for 30 minutes at RT. (Perform all rinses in Dark Box, or keep tubes wrapped in foil.) Replace the rinse solution with fresh rinse. Repeat 5 times total, for 2.5 hours. Perform a final 30 min rinse into PBSTween.

9. *Dissect*. Move embryos to a dissection scope and prepare for mounting. For central canal imaging, dissect head and yolk away.

10. *Prepare slide mounts*. Place 2 small pieces of electrical tape on a slide, approximately 1 cm apart. These will act as risers, and prevent embryos from being flattened. Once embryos are ready, drop one ~30 uL drop of Aquamount between the two pieces of tape. Transfer embryos into Aquamount, in as little additional liquid as possible. Manipulate and position embryos as desired, using an eyelash or forceps. Carefully apply a clean coverslip. Tilt slide slowly, to allow Aquamount to fill entire coverslipped area.

    Check position of embryos under a dissection scope, and gently roll coverslip to achieve lateral positioning of most embryos. Once fish are positioned, seal edges of coverslip with nail polish, and allow the slides to cure FLAT in a dark place, for at least 12 hours.

Note: Aquamount ostensibly hardens, which should prevent slide leaking— but that has not been my experience. I've found that I cannot store slides tilted onto one side, no matter how long I've let them set… they will leak and be ruined. So, keep slides horizontal until they are ready to be imaged, AND keep them horizontal during longterm storage.

Recipes:

PBS
1x PBS, prepared with DI Water, from 10x Sigma Stock Solution

4% PFA in PBS
1 Ampule of 20% PFA, diluted in 80 mLs of PBS
Aliquot 1mL into 1.5 mL Eppendorf Tubes, store at -20 °C.
When needed, thaw at 37 °C, vortexing briefly if precipitate has formed.

PBST
Only required for Alternative Step 3B.
0.5% Triton-x100, diluted in 1x PBS

PBSTween
0.1% Tween-20, diluted in 1x PBS

Blocking Solution
5% Normal Sheep Serum (NSS)
1% DMSO
Diluted in PBSTween

Note: This is a pretty minimal blocking solution, for antibodies with high affinity. (Tubulin antibodies stain very specifically, virtually no background.) For unvalidated antibodies, 10% NSS should be used in the initial blocking step.

In general, blocking should be performed with a serum from the host that your secondary is raised in. Thus, NSS may be replaced with donkey or goat serum in different protocols, if you're experiencing high background. This one works great in our hands, though.

Primary Antibody Diluent
1% Normal Sheep Serum (NSS)
1% DMSO
Diluted in PBSTween

Rinse Solution
1% Normal Sheep Serum (NSS)
1% DMSO
0.1M NaCl
Diluted in PBSTween

Example image:

32 hpf larva, mounted laterally and imaged with Zeiss LSM 880: