Talbot Lab antibody labeling protocols for muscle

This document gives three different methods for antibody labeling, which are each suited for different antibodies.

Most antibodies that work with the PFA-no-MeOH also work with PFA-MeOH.
PFA-no-MeOH is preferred when also examining GFP because no further antibody lable is needed for GFP
If using PFA-MeOH and examining GFP, you will need to add anti-GFP antibodies

In our experience, the antibodies that work well with PFA fixation work miserably with Carnoys fixation and vice versa

The Carnoys and PFA-MeOH protocols are largely derived from Bird, 2011
The no-MeOH protocol was developed while I was a grad student at the University of Oregon

Embryo preparation:

Embryos should be fixed in PFA-with-methanol, PFA-withOUT-methanol, or Carnoy's fixative, depending upon the antibody to be used. Each fixation style requires a somewhat different protocol.

PFA fix with Methanol allows for long term storage of embryos
PFA fix without methanol allows for imaging of transgene fluorescence and allows for phalloidin staining.
Carnoy's fixative also allows for long term storage, however I find that subcellular structures are less clear with this fixative.

PFA-MeOH fixation:

1. Raise embryos to desired stage
2. Dechorionate embryos (prior to reaching desired stage)
3. Fix two hours in 4% PFA 1X PBS
4. was 3X in PBST
5. Replace with 100% MeOH
6. Store at -20˚C for up to several months, but at minimum 5 mins.

Carnoy's Fixation

1. Raise embryos to desired stage
2. Dechorionate embryos (prior to reaching desired stage)
3. Fix two hours in Carnoy's fixative
4. Place directly at -20˚C without washing (for at least 5 mins; can be left in freezer for months)

PFA fix, NO METHANOL:

1. Raise embryos to desired stage
2. Dechorionate embryos (prior to reaching desired stage)
3. Fix two hours in PFA (or overnight at 4˚C)
4. Proceed IMMEDIATELY to staining day 1.

Staining protocols for PFA-MeOH and Carnoys fixed embryos

PFA-MeOH fixed fish Day 1:

1. Remove embryos from -20˚C freezer
2. Wash in 75% MeOH/PBST
3. Wash in 50% MeOH/PBST
4. Wash in 25% MeOH/PBST
5. Wash in PBST

Carnoy's fixed fish Day 1:

0. Remove embryos from -20˚C freezer
1. Wash in 95% Etoh
2. Wash in 75% Etoh
3. Wash in 50% Etoh
4. Wash in 25% Etoh
5. Wash once in PBST

Both protocols, Day 1:

6. Wash 2X in PBST-B
7. Wash in PBST-B-N
    a. Bird 2011 recommends blocking 10 mins; I block for a couple of hours
8. Pre-dilute 1˚ antibodies in PBST-B-N
    a. Allow these primaries to pre-absorb at least 20 minutes.
9. Prior to Primary addition, it may be prudent to transfer embryos to 8 well strips, especially if only a few fish per well will be used, and multiple wells use the same antibody combinations. This will conserve antibody, and hasten the protocol.
10. Add primaries to appropriate tubes (0.4ml for epis, 100µl for strips)
11. Place on a shaker at 4˚C overnight

Both protocols Day 2:

1. SAVE ANTIBODIES for reuse (allegedly: better the second time)
2. Wash 1X in PBST-B-N
3. Wash 3X in PBST-B, quickly
4. Wash 3X in PBST over one to two hours (10 mins/wash minimum)
5. Prepare 1:800 dilutions of secondary, in PBST-B-N
    a. Allow secondaries to pre-absorb into block at least 20 minutes
6. Add secondary antibodies to embryos.
7. Rock 4 hours at room temp
8. Wash 4-6X in PBST over an hour or two
9. Store embryos at 4˚C

Protocol for antibody staining PFA-fixed fish without MeOH

Day 1:

1. Fix 2 hours at room temp or overnight @ 4˚C in 1X PBS/4%PFA
2. Wash 3X in PBST
3. Wash in K-block for two hours to overnight (I typically do 4 hours)
4. Prepare primary antibodies in block
    a. Allow primaries to pre-absorb at least 20 minutes prior to addition
5. If desired, transfer embryos into 8 well strips.
6. Add primary antibodies to embryos (0.4ml for epis, 100µl for strips)
7. Shake overnight at 4˚C

Day 2:

8. Remove antibodies, and save for reuse
9. Wash the fish once in K-block or PBST-B-N
10. Wash the fish 3X quickly in PBST or PBST-B
11. Wash the fish 3X over one to two hours in PBST
12. Prepare secondary antibodies: 1:800 in K-block or PBST-B-N
    a. Allow secondary antibodies to pre-absorb at least 20 minutes
13. Add secondary antibodies to embryos (0.4ml for epis, 100µl for strips)
14. Rock four hours at room temp. Alternatively, rock overnight at 4˚C
    After this, keep the fish in the "dark" (tinfoil)
15. Wash the fish in PBST several times over an hour or two
16. Store the fish at 4˚C.

Phalloidin staining:

CAUTION: phalloidin is toxic. Use gloves, and dispose in appropriate waste bins.

Do phalloidin staining after step 11 of day 2.

1. Prepare Phalloidin dilution (1:20 in PBS)
2. Wash embryos 2X quickly in PBS
3. Add 1:20 Phalloidin-546
4. Rock 1 hour at room temp- in tinfoil.
5. Wash 3X in PBS
6. Rock overnight in PBST
7. Wash a few times in PBST
8. Store at 4˚C

Recipes:

20% Tween-20
___Add 40ml sterile H2O to a conical ___fill to 50ml w. tween-20
___mix vigorously

20% Triton-X
___Add 40ml sterile H2O to a conical ___fill to 50ml w. Triton-X
___mix vigorously

PBST (1 L): 1X PBS, 0.1% Tween-20
___ 100ml 10X PBS ___5ml 20% tween-20 ___ fill to 1L with Sterile H_2O

PBST-B (50ml): 1X PBS, 0.1% Tween-20, 2% BSA
___50ml PBST ___1g Bovine Serum Albunim (BSA) powder
___ mix well to dissolve BSA

PBST-B-N (50 ml): 1X PBS, 0.1% Tween-20, 2% BSA, and 5% NGS
___47.5ml PBST ___1 g BSA powder _
__2.5ml Normal Goat Serum (NGS) ___Mix well to dissolve BSA

K-Block (10 ml): 1XPBS, 0.5% TritonX-100, 4% NGS 2% NSS 1% DMSO
___1ml 10X PBS ___50µl 20% Triton-X ___100µl DMSO
___400µl NGS ___200µl Normal Sheep Serum (NSS)
___8.3ml Sterile H_2O

Carnoy's fixative (5 ml): 60% Etoh, 30% Chloroform, 10% Glacial Acetic Acid
___ 3ml 100% Etoh ____1.5 ml Chloroform ___0.5 ml glacial acetic acid

PFA fixative (10 ml): 1X PBS, 4% PFA
____ 1ml 10X PBS ____4ml 8% PFA (<1 week old) ____5ml sterile H_20