Rhodamine-Phalloidin Staining

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This procedure is taken from http://www.cooperlab.wustl.edu/Methods/PhalloidinStain.html

1. Rinse cells or tissue in PBS briefly to remove media components.

2. Fix cells or tissue in 1-2% paraformaldehyde in PBS (freshly prepared) for 15 min at RT. The time and concentration of fixative can vary, depending on the thickness of the specimen (time for fixative to diffuse into the sample) and on how sensitive the antigen may be to the fixative. For an unknown sample, it is worthwhile to vary the conditions widely in a pilot experiment.

Prepare 10% para. stock like this: 1g paraformaldehyde in 10 ml dH_2O Heat to ~60°C Add 1 drop of 1M NaOH and continue heating until most is dissolved. Filter stock thru 0.2 µm filter

*** Phalloidin binding requires the F-actin to have a protein structure near native. Methanol or acetone used to fix and / or permeabilize essentially abolishes phalloidin binding. ****

3. Quench excess aldehyde with 10mM ethanolamine in PBS (or 0.1 M glycine in PBS) for 5 min.

4. Permeabilize cells in 0.1% Triton-X100 in PBS for 1 min.

5. Incubate cells in Rhodamine-phalloidin (Molecular Probes) diluted 1:100 in PBS for 15 min.

6. Rinse 3 times in PBS, 5 min/wash.

(Alternative: For vertebrate cells, whose actin binds phalloidin very tightly, one can add Rh-ph to the final PBS wash at 1:1000 and then simply mount in that medium

7. Mount for microscopy using PBS-buffered 50% glycerol as mounting medium. Use an anti-bleaching agent, *p*-phenylene diamine or N-propyl gallate. (I would not use glycerol unless necessary for some other reason. PBS should be fine.)

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To see my own procedure, specified for *S. mansoni* cercaria, schistosomula and adult stages, **go to the second page.**

Confocal Immunofluorescence using PFA as a fixative

(1) Prepare **1N NaOH** (0.4g/10ml water).
(2) **Quench** excess of PFA: Prepare **0.1M Glycine** in PBS (i.e. 0.75g/100ml). This will be used to quench the excess PFA after fixation.
(3) **Permeabilization**: 0.5% Triton X 100 in PBS.
(4) **Blocker**: 5% Goat serum, 0.5% Triton X 100 in PBS.
(5) Prepare **10% PFA** by dissolving 1g PFA in 10ml water and heat at 60°C in the fume hood. Complete dissolve PFA by Adding 1drop of 1N NaOH. Filter stock 0.2µm filter.
(6) Prepare 25ml of **4% PFA** in PBS: 10ml of 10% PFA + 12.5ml H2O + 2.5ml of 10X PBS.

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(A) Rinse cultured schistosomula animals twice with PBS to get rid of culture medium components. Spin for 10min at 1000-12000 rpm (using the bench centrifuge in the culture room).

(B) **Fix** the animals with **4% PFA** (in PBS) for 3-4hours at 4°C, using end over end rotor.

(C) Wash the animals twice in PBS and **quench the excess of PFA with 0.1M Glycine** (0.75g in 100ml PBS) for 5-10min. This will reduce auto-fluorescence. Rinse/spin twice.

(D) Block with the **blocker** for 24hr at 4°C, using end-over-end rotor.

(E) Wash/spin twice with PBS and 1200rpm/10min.

(F) In separate tubes, add **1:100** precleared polyclonal **Rabbit anti SmGPCR-il3 IgG**. Precleared the antibody with **1:25** of 4µg/µl bacterial lysate or precleared/preadsorbed IgG (preadsorbed with **1:31.25 recombinant** il3 of 0.47 µg/µl. Make the 1ry Ab, bacterial lysate and recomb. il3 protein in blocker solution and incubate for 2-3days at 4C, using end-over-end rotor.

(H) Wash 3-4 times with PBS. Spin for 5-10min at 1200**0**rpm (using small centriguge in our main lab).

(I) Add **1:300 2ry Ab-FITC** (Chemicon), use 6.666µl per 2ml of the blocker and fraction it into 4 fractions, each is 500µl. Wrap the tubes in aluminum foil to reduce the excitation of the conjugate dye and incubate for 2days at 4C, using end-over-end rotor.

(J) if pholloidin is needed to be added (to study the muscular tissue or as a counter stain), add it next day of adding the 2ry Ab and without washing of the antibody. Incubate ***200ng-400ng/ml Phalloidin-TRITC** (i.e. **1:1000-1:500**) for 24-48hours after wrapping the samples at 4C, using end-over-end rotor.

(K) Wash 3-4 times with PBS, 15min incubation/washing and 10min spinning

(L) Mount 1drop of antifade mounting medium + 50-100µl of pellet on a slide, add the cover slip and seal the edges with nail polish.

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Primary Ab preparation:

In 2ml of blocker (5% Goat serum, 0.5% Triton X 100, in PBS), add 20 µl of IgG (**1:100**) and 80µl of (4 µg/µl) bacterial lysate (i.e. **1:25**). Divide into two halves. Label first half (1ml) as precleared IgG and in the second half, add 32µl of 0.47 µg/µl recombinant IL3 (i.e. **1: 31.25**).

Phalloidin staining:

Phalloidin-TRITC (Sigma P-1951) is supplied as 100µg. I reconstituted it in 500µl of ddH_2O to obtain stock concentration of 0.2mg/ml (or $200x10^3$ng/ml). The recommended dose is 200ng/ml (Mair et al, 2003). Thus, I can use 1:1000 of that prepared stock, however, I used 1:500 (i.e. 1µl of the stock per 0.5ml blocker).