FMRFamide STAINING PROTOCOL by Chris Li

- Grow at least 1 big plate of happy worms (ie. well-fed-sho uld not be starved)
- Wash worms with M9 into a 15 ml Falcon tube, spin @1700 rpm for 1 min in a clinical centrifuge.
- Wash 1x to get rid of bacteria.
- Fix in 4%paraformaldehyde (best is EM grade: 16%paraformaldehyde Electron Microscopy Sciences Cat No: 15700, kept @4°C) in pH7.4, 0.1 M PB (phosphate buffer) in a volume of; fixative/worms =10/1 (vol/vol), 12-36 hr @ 4°C on a nutator (decrease fixation time if antigen is sensitive to being over-fixed. Fixation times have not been a problem with anti-tubulin, anti-NCAM, anti-serotonin, anti-myosin or anti-FMRFamide antisera)

0.1M Phosphate Buffer pH 7.4

77.4 ml from 1M Na2HPO4
22.6 ml from 1M NaH2PO4
add ddH20 upto 1L

4% paraformaldehyde in PB
3 ml PB
1 ml 16% paraformaldehyde

- Rinse 6x with 0.1 M phosphate buffer, pH 7.4 (worms can be stored in PB several days @ 4°C
- Incubate animals in 1 ml 5%beta-mercaptoethanol (beta-ME),1% Triton-X-100 in 0.1M Tris pH 6.9, 24-48hr @ 37°C (or pH 7.2 @ room temp) on a nutator.

200 ul 10 %Triton-X-100
100 ul beta-ME
200 ul 1 M Tris , pH 7.2
1500 ul ddH2O

- Rinse with PB until you can't smell beta-ME (worms can be stored in PB for a few days @ 4°C)
- Rinse 1x with 0.1 M Tris pH 7.7 (room temp)

- Add 1 ml of 900 U/ml type IV collagenase in 1mM $CaCl_2$ in 0.1 M Tris pH 7.7 @ room temp (or pH 7.4 @ 37°C). Transfer to an eppendorf tube and incubate @ 37°C for 1 hr 15 min (no longer! However incubation time must be checked with each new batch of collagenase; it can be incubated 1-15 hr, after 1 hr, check every 30 min. When you see a few broken animals and some eggs floating around the incubation must be stopped immediately by transferring the tube onto ice)

Collagenase solution
100 ul 1 M Tris pH 7.7
Appropriate amount of freshly prepared (900 U) collagenase stock in ddH_20
1ul 1M $CaCl_2$
upto 1 ml ddH2O

- IMPORTANT; AFTER THIS STAGE ANIMALS BECOME QUITE FRAGILE SO THE SPINS MUST BE DONE IN THE MICROFUGE @ 1700-2000 rpm WITH SLOW ACCELERATION/DECELERATION.

- Rinse 2-3x with PB. (worms can be stored in PB for a few days @ 4°C).

- Incubate with 10% goat serum, 0.5% Triton-X-100 in PB (Blocking sol) for 30 min-3 hr, @ 37°C.

Blocking solution
100 ul goat serum (GIBCO)
50 ul 10% Triton-X-100
0.2% (by wt) sodium azide
upto 1 ml PB

- Incubate with 50-100 ul rabbit anti-FMRFamide (or any other primary antibody)1/500 +mouse anti-GFP (Molecular Probes mouse anti-GFP 3E6) 1/100 in blocking sol overnight @ room temp.

- NOTE: If your primary is monoclonal you must use polyclonal anti-GFP antibodies, and change the species of the secondaries accordingly. Some antibodies are more sensitive to high amounts of Triton or to overnight room temp. incubation than others. You may need to decrease Triton to 0.1% or incubate the primary antibody @ 4°C.
- Rinse 2-3x with blocking solution.
- Incubate in 75-100 ul secondary antibody sol: FITC goat anti-mouse 1/100 and Cy3 goat anti-rabbit 1/100 in blocking sol, for 1-3 hr.s @ room temp.
- Rinse 3x with ddH_2O.
- Withdraw as much water out as you can without sucking the pellet out. Add 1/2 drop of Slowfade â component A and 0.2 ul (not more!) DAPI (NB** the pellet must be thoroughly washed with ddH_2O to get rid of buffer). Cut the very tip of a 200 ul pipette tip to have a bigger pore. Pipette the animals onto a frosted side slide, cover with coverslip and fix the sides with nail polish. Check the slides freshly stained. You can keep the slides for a couple of years at 4oC provided they are protected from light.