Protocol : Finney-Ruvkun fixation for immunostaining

This method work well for many antibodies. Taken from Bettinger et al., 1996, Development 122 : 2517-2527

The key to success for good staining is to the fixed worms as gently as possible. It is also better not to try to take off all the supernatant after h incubation/wash

Growth the worms you need. Make about 30-50 plates because you can store the worms at the end.

Part 1 (about 1 hour)

- Place a 1ML of **2x RFB** in a ice/water bath
- Wash the worms off the plates with M9 into 15ml Falcon tube
- Make 3 washes over the course of about 45 mins (no mor bacteria)
- After the final M9 wash, do one more with 5ml water
- Remove s/n and then adjust the total volume of the sus nsion to 900µl. Do not transfer the worms
- Place the worms suspension into ice/water bath for at least 5min to cool. Label the tube
- While the worms are cooling, prepare some liquid nitro n
- Add 1ml of ice-cold **2x RFB** to the cooled worm suspension and mix gently
- Add carefully 110µl of 36.5% **formaldehyde** to the worms and mix gently again
- Parafilm around the top to seal it and the immediately nap freeze the worms in the liquid nitrogen
- The frozen worms can be conveniently stored at – 80°C. If the worms are to be used immediately, they must still be frozen in liquid nitrogen before the next stage. The freezing help to permeabilize the cuticle t low antibody access.

Part 2 (about 9 hours)

- Remove the worms from the -80°C freezer and thaw them under a stream of warm water. Don't let the worms themselves warm up. As soon as the suspension has thawed, mix it gently and then place it into an ice/water bath

- Incubate the worms in the ice bath for **3h30**. Gently mix the worms by inverting the tube several times every 30min. Don't mix after the final 30min

- Spin down in a cooled centrifuge at 1000rpm for 1min. ove the s/n and wash twice at RT with 2ml of **TTE**.

- After the second wash, remove the s/n and add 2ml fres ly prepared **TTE +1% mercaptoethanol** (20µl of pure mercapto into 2ml) mercaptoethanol and DTT reduce disulfide linkages that help hold the cutic gether. Triton keeps the worms from sticking to each other. The disulfide r ction is complete within minutes. The extended incubation at 37°C is to l worm enzymes like DNases, proteases and peroxidases which interfere with the oxidation step

- Incubate the worms in a 37°C water bath for **4h**. Mix gently as before every 30min

- Don't mix the worms after the final 30min but spin them down at 1000rpm for 1min. Remove s/n and the wash the womrs once with 2ml of **1xBO_3 buffer** at RT. Spin, remove the s/n and add 2ml of freshly prepared **1xBO_3 +10mM DTT**. Incubate in a 37°C water bath for 15min

- Spin, remove s/n and then wash with 2ml of **1xBO_3** buffer. Spin again, remove s/n and the and 2ml of freshly prepared **1xBO_3 + 0.3% H_2O_2**. Incubate for 15min at RT. The timing of this last step is critical. The worms will be destroyed if they are left in this solution for too lo g. It is also important that the H_2O_2 is not too old. You should be able to see bublles forming in the tubes after about 5min or so.

- When the 15min are over, pellet the worms by spinning as before. Remove s/n and then wash with 2ml of **1xBO_3.** Spin again, remove s/n and add 3ml of **PTC**. Leave the worms to rock GENTLY at RT for 30min. Spin down, remove s/n anda dd **PTB** to a final volume of 2ml. You should have around 100-125µl of packed worms. These worms are stable for several mo h if kept at 4°C

Staining:

- Spin the fixed worms in PTB to the bottom of the tube

- Transfer 40µl of packed worms with a cut off yellow tip into a ED

- Add 160µl of PTB

- Add the antibody/ies with correct final dilution (1/1000 for MH27, 1/500 for anti-β-Gal AB)

- Rotate on wheel @4°C o/n

- Spin down the worms. Remove s/n, wash with 1ml of PTC, remove immediately

- Wash 3x with 1ml PTC for 1 hour of incubation (can make 3 washe 5min and the 3rd for 2 hours)

- Final wash with PTB (no incubation)

- Spin the worms and remove s/n. Should have about 40µl of worms

- Add 160µl of PTB + second antibody (1/1000 for goat anti-mouse-Cy3). Should be in the dark !!!, Cy-3 is photo-reactive (aluminium sheet around the ED) Here 2 possibilities ;

    - O/n @ 4°C
    - 3 hours @ RT

- Spin and remove s/n. 5 washes with 1ml of PTC – the first immediate and the other four 1 hour (same as before, but there is more background)

- Remove s/n

- Keep the worms in the dark @ 4°C for months in 100-200µl of PTC

- 3µl of worms are mounted on slice

- Option : DAPI staining : Make DAPI/Vectashield on the slice (see protocol)

Buffers :

2x RFB for 50ml

160mM KCl
20mM EGTA
30mM PIPES
40mM NaCl
10mM spermidine
50% Methanol

Mix in a tube 5ml of 0.2M EGTA (pH about 8), 500µl of 1M spermidine, 5ml of 0.3M PIPES (add NaOH to dissolve, final pH about 6), 8ml of 1M KCl and 2 ml of 1M NaCl

Carefully pH the solution to 7.4 with HCl. Adjust the volume to 25ml with water and add 25ml of methanol. After addition of methanol the pH will be approx. 7.25. Store @4°C

TTE for 100ml

100mM Tris-HCl ph 7.4 1mM EDTA
1% (v/v) Triton X-100

Mix 10ml of 1M Tris-HCl pH 7.4, 200µl of 0.5M EDTA and 79ml of water. Add 10ml of Triton X-100 10% solution

100x BO_3 buffer
1M H_3BO_3 pHed to 9.2 with NaOH

10mM DTT in 1x BO_3 (2ml needed per tube)

Make 1 :100 dilution of the 100x BO_3 buffer. To 10ml of the diluted solution, add 15.4 mg of solid DTT (Mw 154g/mol). USE FRESH, do not store !!!

Or

2ml of 1xBO_3 + 20µl DTT 1M

0.3% H_2O_2 in 1x BO_3 (2ml needed per tube)

2ml of 1x BO_3 + 100µl of 30% H_2O_2. Use fresh

PTB

1x PBS 1% BSA
1mM EDTA 0.5% Triton X-100
0.05% sodium azide

First make up 1x PBS :

Disolve : 8g NaCl,
0.2g KCl
1.42 anhydrous Na_2HPO_4 (1.75g if dihydrat)
0.24g KH_2PO_4
in 800ml water
Adjust p£H to 7.2 with HCl 1M. Adjust volume to 1L

It's not the same pH if you use PBS 10x !!!

For 50ml PTB: 48.25ml of 1x PBS
0.5g BSA

100µl of 0.5M EDTA
1.25ml of 2% natrium azide
When BSA is disolved add 250µl of Triton-X 100. Stire and keep at 4°C

PTC

PTC is the same as PTB but 0.1% BSA

For 200ml PTC : 193ml 1x PBS
0.4ml of 0.5M EDTA
5ml sodium azide
0.2g BSA
1ml Triton X-100