Antibody Staining of Formaldehyde-fixed Worms by Gary Ruvkun and Michael Finney and **Modified Finney- Ruvkun protocol**

This protocol works for all stages except dauers (which will not open) and hypochlorite-treated eggs (which disintegrate).

1. Wash animals with a few mls H2O into an eppendorf tube. Let animals settle, pour off supernatant. Wash again with H2O. Spin at 2000 rpm (with slow acceleration/decelaration) for 2 min, gently aspirate off supernatant.

2- Prepare fixative:

1.0 ml 2X MRWB

200 ul 10% formaldehyde (1% final)

800 ul ddH2O

3- Fixation: Add 1.25 ml fixative per tube, mix well by gentle inversion.

4- Immerse the tube in dry ice/ethanol to freeze (at this point frozen worms may be stored at -80oC to be used later). Thaw the tube contents on ice and incubate on ice with occasional agitation for 1/2 hr to overnight.

5- Reduction: Spin worms at 2000 rpm for 2 min, gently aspirate off supernatant. Wash worms twice with 1X TTB. Resuspend worms in 1 ml 1X TTB + 1% b mercaptoethanol (b mercaptoethanol and DTT reduce disulfide linkages that help hold the cuticle together. triton keeps the worms from sticking to each other. The disulfide reaction is complete within minutes. The extended incubation at 37oC is to kill worm enzymes like DNases, proteases and peroxidases which interfere with the oxidation step). Incubate at 37oC for 1.5-2 hr with gentle shaking. After this point the worms are fragile and should not be spun hard.

6- Spin worms at 1500-2000 rpm for 2 min, gently aspirate off supernatant.

7- Resuspend in 1 ml 1X BO3 buffer. Spin worms down and aspirate off supernatant.

8- Resuspend in 1 ml 1X BO3 buffer + 10 mM DTT. Shake gently for 15 min.

9- Oxidation: Spin worms and gently aspirate off supernatant. Resuspend in 1 ml 1X BO3, spin down worms and remove supernatant. Resuspend in 1 ml 1X BO3 buffer + 0.3% H2O2 (H2O2 oxidizes the -SH groups to -SO3. Do not overwash so disulfides do not reform. BO3 buffer provides the basic pH needed for the reaction. Cysteins and methionines in other proteins will be oxidized as well, possibly affecting someepitopes. Met but not Cys can be restored by a second DTT treatment). Shake at room temp for 15 min but keep tubes upright since the caps may pop open from O2 pressure (wrapping the tops in parafilm may help).

10- Spin worms and gently aspirate off supernatant. Resuspend in 1 ml 1X BO3 buffer. Spin worms down and gently aspirate off supernatant.

11- Resuspend worms in 1 ml Ab B for 15 min. Spin down at 2000 rpm for 2 min. Aspirate off supernatant and resuspend in 1 ml Ab A. At this point you can store worms at 4oC in Ab A.

12- Staining: Transfer 15-20 ul aliquot of fixed worms to a microfuge for staining. Add an appropriate dilution of antibody (start with 1/10 to 1/1000) in 200-500 ul Ab A. Incubate at room temperature for 2 hr or overnight at 4oC (gently shaking and wrapped in aluminum foil if fluorescently tagged probes are being used).

13- Spin worms at 1500-2000 rpm for 2 min, gently aspirate off supernatant.

14- Resuspend in Ab B and repeat spin. Aspirate off supernatant. resuspend in Ab B and shake gently at room temp for 2 hr.

15- Spin worms at 1500-2000 rpm for 2 min, gently aspirate off supernatant.

16- Add an appropriate dilution of fluorescently tagged secondary antibody (start with 1/100 dil) in 200-500 ul Ab A. Incubate at room temperature for 2 hr or overnight (gently shaking and wrapped in aluminum foil).

17- Spin worms at 1500-2000 rpm for 2 min, gently aspirate off supernatant.

18- Resuspend in Ab B and spin at 1500-2000 rpm for 2 min, gently aspirate off supernatant. Resuspend in Ab B and shake egntly at room temp for 2 hr.

19- Repeat spin, remove supernatant, resuspend in Ab A, repeat spin, remove most of supernatant.

20- Mount worms for observation: Add 1/2 drop of SlowfadeÇ component A and if wanted, 0.2 ul DAPI . 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.

SOLUTIONS:

2X Modified Ruvkun's Witches Brew (MRWB)

800 ul 2 M KCl (160 mM final)

80 ul 5M NaCl (40 mM final)

2.0 ml 0.1M EGTA (20 mM final)

1.0 ml 0.1M Spermidine (10 mM final)

600 ul 0.5M PIPES, pH 7.4 (15.1 g/100ml)-goes into solution at pH 7.4

5.0 ml 100% Methanol (50% final)

ddH2O up to 10 ml

Methanol precipitates proteins, reducing diffusion before cross-linking. Spermidine and formaldehyde together cross-link proteins. Formaldehyde concentration and fixation time are important and should be optimized-1% for 1/2 hr is a good start. More fixation will stabilize some antigens but destroy others (like ***unc-86***).Freezing cracks egg shells, letting the fixatives in.

Tris-Triton Buffer (TTB):

1.0 ml 1 M Tris HCl, pH 7.4

100 ul Triton-X-100

20 ul 0.5M EDTA

ddH2O up to 10 ml

40 X Borate Buffer (pH 9.2):

618 mg H3BO3

5.0 ml 1M NaOH (doesn't go into solution unless NaOH is added)

ddH2O up to 10 ml (pH 9.2, heat to dissolve)

Antibody buffer A (Ab A):

2.0 ml 5X PBS

5.0 ml 2%BSA

50 ul Triton-X-100

250 ul 2% NaN3

20 ul 0.5M EDTA

ddH2O up to 10 ml

Antibody buffer B (Ab B):

Ab A + 0.2%BSA

10% Paraformaldehyde:

1ml PBS

0.5 ul 10N NaOH

0.1 g paraformaldehyde

heat at 65oC to dissolve.

Modified Finney Ruvkun protocol

(Also see: Bettinger J.C. et al 1996)

1- Wash worms well (5 times) with M9 and then once with RFB + 2% formaldehyde.

2- Add 1 ml RFB + 2% formaldehyde to worms.

3- Freeze in dry-ice/ethanol, thaw. Put on ice/icewater bath for 3.5 hr with occasional inversion.

4- Wash once with TTB.

5- Put worms in TTB + 1% b-mercaptoethanol at 37oC with gentle agitation for 4 hr.

6- Wash once with BO3 buffer.

7- Incubate in BO3 buffer/10 mM DTT at 37oC with gentle agitation for 15 min.

8- Wash once with BO3 buffer.

9- Incubate in BO3 buffer/0.3% (v/v) H2O2 at room temp with gentle agitation for 15 min.

10- Wash once with BO3 buffer and incubate in Ab B for 30 min at room temp.

11-Spin worms down gently and put in Ab A (at this step you can store worms at 4oC for several months).

12- Incubate worms in primary antibody in Ab A at room temp for 8-12 hr or at 4oC overnight (suggested dilutions for Clontech rabbit anti-GFP is 1/200 dil. and mouse anti MH27 is 1/1000 dil).

13- Wash worms in Ab B for several changes for 4 hr at room temp.

14- Incubate worms in secondary antibody in Ab A (suggested dilution: 1/300) at 4oC overnight.

15- WWash worms in Ab B for several changes.

16- Mount worms on agar pad as described above.

Optional DNA stains:

DAPI: Can be added 1 mg/ml during one of secondary antibody washes (approximately 15 min at room temp)

TOTO-3 (Molecular Probes): Add to secondary antibody incubation as 2uM or first wash of secondary antibody for 1 hr at room temp.

SOLUTIONS:
2X RFB (Ruvkun Finney Buffer): (10 ml total)

1.6 ml 1M KCl

80 ul 5M NaCl

0.4 ml 0.5M EGTA

0.6 ml 0.5 M PIPES

0.1 ml 1M spermidine

5 ml Methanol

2.22 ml ddH20

RFB + 2% formaldehyde: (4 ml total)

2 ml 2X RFB

1.78 ml dd H2O

216 ul 37% formaldehyde

TTB: same as above

40 X Borate Buffer (pH 9.2): same as above

Antibody buffer A (Ab A): same as above

Antibody buffer B (Ab B): same as above