FREEZE-CRACK & STAINING PROTOCOL by Janet Duerr

NOTE: THIS STAINING WORKS WELL WITH GFP ANTIBODIES

First Day: Freezing Worms

Use a plate of unstarved worms (starved worms are more autofluorescenct; dauers don't crack)

For same day fixation with methanol and acetone (VAChT, VMAT, ChAT, UNC-18, UNC-13, etc.):

Put (slightly **toxic**) acetone and methanol into staining dishes in ice

For same day hard fixation (SNT-1, UNC-41, neurotransmitters, etc.):

Prepare **TOXIC** formaldehyde and / or glutaraldehyde buffer (usually on ice)

Place large flat slabs of dry ice in a Styrofoam container

Wash the worms from the plate with M9 buffer and collect them in a 1.5 ml tube

Spin the worms at 2000 rpm for 2-3 minutes (or to collect mainly adults, let settle on ice)

Pipet off the supernatant and repeat M9 rinse

If supernatant is not perfectly clear (due to remnant bacteria), do another M9 wash

Rinse worms with dd. water, spin or let settle, and remove all but about 50-150 ul water

Label lab-made poly-lysine slides with indelible ink and place on counter frosted side up

(2-3 slides per tube)

Using a glass pipet, place a drop of worms on the slide, let the worms settle briefly, then spread the worms on the slide with the side of the glass pipet

Carefully press an unlabeled (commercial) poly-lysine slide down on the worms label side down so that only the unfrosted parts overlap

Press straight down hard enough to immobilize the adults without rupturing most of them

Carefully (without sliding) put the slides on dry ice for at least 20 minutes

At this point, you can save the slides at -80^{o}C (with possibly some decrease in staining). Let slides from -80^{o}C 'warm up' on dry ice for 20 min. before cracking

First Day or Later: Fixing and Staining Worms

Have fixative ready

Crack the slide sandwich by swiftly pulling apart the two slides; discard the top (unlabeled) slide

For methanol / acetone 'fixation' (when possible, keep these slightly **toxic** fixatives covered):

Immediately place the wormless sides of the slides (smooth sides) back-to-back

Immediately place the slides in a cold slide holder

Immediately immerse in ice cold methanol for 2 minutes

Immerse in ice cold acetone for 4 minutes

Air dry the slides for less than a minute or blot edges on paper towels

Place the slides in a coplin jar (wear gloves)

Rinse the slides briefly in PBS (do not pour PBS directly onto worms, they'll wash off)

(Filter the acetone and methanol and save for reuse)

For harder fixations (do in hood or keep these **TOXIC** fixatives well covered):

For individual slides, quickly place the slides (worms up) flat in a dish and cover the worms with 1-4% formaldehyde + / - 0.1-1.0% glutaraldehyde in buffer

Or, place the slides in a coplin jar with 1-4% form. + / - 0.1-1.0% glut. in buffer

Keep worms in fix for 30 min.- 24 hr at room temperature or in the cold / on ice

Rinse gently several times with PBS (until the slides no longer smell of fixative)

Put the slides into a dish with the proper block for 1 hour (room temp) or overnight (4^{o}C)

Filter and save block

For large volumes of Ab (best staining):

Pour 1^{o} Ab (straight supernatant or PAb at 1:50-1:1,000) into coplin jar

Incubate overnight at 4^{o}C on bench or with gentle shaking

For small volumes of Ab (staining will not be uniform):

Have primaries made up and ready

Set up plates for slides: 96-well plate lids with dd. water along the two short edges

Dry outer edges and back of slide with a kimwipe

To use only a little antibody, draw a circle around the worms with a wax/grease pencil

Put the slides in the 'dish' (slides must not be in contact with water or each other)

Put on primary (1-600 ul per slide) before worms dry out and cover 'dish' with another lid

Leave overnight on counter at 4^{o}C or room temperature

Second+ Day of Staining:

Pour off and filter and save the primary antibody, if desired

If labeled individually, rinse slides gently with PBS from squirt bottle and put into coplin jar

If already in jar, pour in, then immediately pour out, PBS

Rinse 2-3x (~30 min. each) in Ab buffer

Put in secondary antibody (e.g., 1:100 fluorescent goat anti-mouse in Ab buffer with serum)

Cover well to protect from light

Let sit 4 hours at room temperature (MAb or PAb) or overnight at 4^{o}C (PAb)

Pour off, filter, and save secondary antibody

Pour in, then immediately pour out, PBS

Wash 2-3x (at least 30 min. each) with Ab buffer, keeping protected from light

Background is better for antibodies with high affinities with the last wash overnight in the cold room (overnight washing may decreases MAb staining)

Change to PBS for at least 5 min.

Mounting Slides:

Be sure no sunlight is shining on the bench

Remove one to four slides from the PBS and place on paper towel

Dry the back of each slide with a kimwipe

Place 2 drops (using pipetman) of **TOXIC** mounting medium over worms so that there are approximately equal parts medium and PBS on slide

Tilt slide to mix the medium with PBS

Gently put down edge of 24X60 mm coverslip on slide using fine forceps or your fingertips

Slowly let coverslip down; try not to let any air bubbles form. If bubbles form, lift the edge of the coverslip slowly, then relower without sliding the coverslip across the worms

Carefully dry off the edge of the slide (without moving the coverslip).

Seal the coverslip with at least three layers of nail polish

After the polish is dry, carefully clean off the outer surfaces of the slide and coverslip with Kleenex

Keep the slide covered (in the dark) and cold when not in use

Reseal the coverslip with more nail polish as needed, e.g., after using any oil immersion lens

Antibody staining persists for months in sealed slides at -20^{o}C; DNA staining fades and spreads

Solutions and Storage:

Methanol and acetone are kept at 4^{o}C

Filter solutions through coarse filter back into bottles

Use ~5 times (until rather discolored)

"Lab-made" Poly-L-lysine slides (kept in boxes in the refrigerator or cold room):

Wipe slides very 'clean' with kimwipes and place back-to-back in slide holder

Shake 30 min. in dilute ionic detergent (e.g., squirt of ivory soap in water)

Rinse in running water > 1 min.

Rinse in running d water > 1 min.

Shake in 70% ethanol + 1% HCl for > 5 min.

Rinse in running d water > 5 min.

Dry in 60^{o}C oven > 5 min.

Shake in poly-L-lysine 5 min.

Dry in 60^{o}C oven > 1 hr to overnight

Wearing safety goggles and working over a pad, crack the slides apart with a scalpel

Store in clean slide box at 4^{o}C until needed

poly-L-lysine solution (kept in refrigerator):

200-400 mg	Sigma P1524 poly-L-lysine
200 ml	dd. water
0.2 g	sodium azide (0.1%) **TOXIC**

Mix together and store at 4^{o}C.

Reuse solution to coat slides until it becomes noticeably less sticky

Commercial poly-lysine slides (Sigma poly-prep) are not nearly as sticky as lab-made slides. They (or silane coated slides) are used for the top slide of the sandwich. Then, less of the worm sticks to the (discarded) top slide.

10X PBS (kept on shelf at room temp). For 1 L:

80 g	NaCl
2.0 g	KCl
27.2 g	Na_2HPO_4:7H_20
2.4 g	KH_2PO_4
2 g	sodium azide **TOXIC**

Allow salts to dissolve (with gentle heat and stirring) in 900 ml dd. water. pH to 7.2 with 10M NaOH. Top to 1 L; autoclave.

Antibody buffer (kept in cold room). For 1 L Ab buffer:

100 ml	10X PBS
5 ml	Triton X-100 (0.5% final)
2 ml	0.5 M EDTA, pH 8 (1mM final)
1 g	BSA (0.1% final)
0.3 g	sodium azide (0.05% final)

Add about 700 ml dd. water to 10X PBS. Add rest of chemicals. pH to 7.2 with HCl. Add dd. water to 1 L. Store at 4^{o}C.

Block (10% serum in antibody buffer) (kept in cold box)

Block should be made with serum from the same species used to generate the 2^{o}

Block must not contain serum from the animal used to generate the 1°

We generally use secondary antibodies that were made by donkeys, so we use donkey serum (available from Jackson ImmunoResearch 800-367-5296)

Filter solution through coarse filter back into bottle. Use 5-10 times or for 1-2 months (until it starts to grows bacteria)

Primary polyclonal antibodies (usually kept in cold box):

Make up 1:50-1:25,000 in Antibody buffer (plus 1% BSA or 10% serum). (Serum is a more effective block, but is expensive and 'goes bad' more quickly than BSA.)

Blocking serum, if used, should be from the same species used to generate the 2°

Secondary antibodies in use are (mostly) stored in dark bottles in the refrigerator

Secondary antibodies must be cleaned (see Cleaning Secondary Antibodies Protocol)

Avoid repeated freeze thawing of antibodies

Make up secondary 1:50-1:500 in Antibody buffer plus block of 1% BSA or 10% serum

If using serum as block, use serum from the same species used to generate the 2°

Keep in dark whenever possible

Secondaries may be used many times (>10), until staining starts to grow dimmer

Cy3 labeled secondaries are highly recommended. They require standard rhodamine filters for viewing and are much brighter. (Jackson ImmunoResearch 800-367-5296)

For double staining, Oregon Green 488or Alexa 488 (Molecular Probes 503-465-8300) use the same filters as fluorescein and resists fading slightly better (but the choice of secondaries from Molecular Probes is limited). We buy dye from Molecular Probes and unlabelled secondary from Jackson lab and couple them ourselves.

Formaldehyde / glutaraldehyde solutions:

To fix neurotransmitters (e.g., GABA) use 4% formaldehyde and 1% glutaraldehyde

Make fresh and use as soon as possible

TOXIC Wear gloves during preparation and use; do not breath fumes

For 40 ml of 4% formaldehyde (e.g.):

Break open a fresh 10 ml ampoule of 16% formaldehyde (Ted Pella 800-237-3526, cat#18505, kept below hood)

Pour into coplin jar in hood (usually on ice)

Add 30 ml 0.1 M phosphate (Na_2PO_4-H_20, pH 7.2) (cold box)

Use as soon as chilled or later the same day

After use, discard down sink with lots of water

Some antigens work better with a mixture of 50% methanol and 1-4% formaldehyde

For glutaraldehyde:

Dilute 25% glutaraldehyde (-20°C) in 0.1 M phosphate or form. solution

After use, discard down sink with lots of water

Mounting Medium ('in use' kept in 1.5 ml tube in film container in refrigerator): **TOXIC**

200 mg	n-propyl gallate
0.3 ml	1 M Tris pH 9
2.7 ml	dd. water
7 ml	glycerol
8 µl	DAPI (**TOXIC**) (2.5 mg / ml in dd. water; stored in 8 ul aliquots in -20°C)

Mix npg, water, and Tris in 15 ml conical tube. Heat at 65^{o}C for about 10 minutes until dissolved. Add glycerol and DAPI and vortex well. Wrap in foil; store in dark at 4^{o}C. Aliquot into 1.5 ml tubes for use (to minimize exposure of the stock to air and light). If it becomes yellow, discard in Biohazard waste and make fresh.

POSTSCRIPT: Freezing Individuals or Small Numbers of Worms

A much larger fraction of fixed worms can be recovered with the following procedure:

Transfer the worm(s) to an unstreaked plate and let them rid themselves of bacteria
Place a small drop of dd water on the middle of a sticky poly-lysine slide
Pick the worms up individually with clean pick and shake them off in the water drop
Multiple worms (up to 20) can be done on the same slide, but they **must** be the same size
(The more worms you do on a slide, the more likely you are to lose some of them)
The worms should partially stick as they fall down in the drop and hit the poly-lysine surface
As described for plates of worms, place a second poly-lysine slide over the worms
Watch carefully while you press the top slide so you compress but don't totally squash the worms
The water droplet should be small so that it does not reach the edges of the slide after compression
Immediately and smoothly transfer the slide to a flat piece of dry ice

With experience and good slides (and luck), 80-90% of the worms can be stained and examined
If any bacteria get into the water, then the worms will not stick as well