Standard Osmium/Gluteraldehyde fixation[a] for *C. elegans*

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Collection of worms.

1. Pick 15 to 30 L4 animals onto a fresh plate. Worms should be healthy and staged and not coming from a chunk. After 24 to 48 hours, pick the worms into 1 x PBS in a glass vial[1] and rinse a couple of times to get rid of *E. coli*. Place glass vial on ice after PBS washes.

Fixation Procedure[2] (plan on ~ 7 hrs for steps 2 to 11 for 3 genotypes ~12 blocks)

2. Remove as much PBS as possible and add at least 300 μl of ice-cold fix A[3].
3. Incubate 1 hr on ice. After the incubation, the worms will look brown (the darker the better). However, there isn't a strict correlation between how dark the worm is and how well the fixation has worked.
4. Four 10mM HEPES washes (done in hood). I put all washes in the osmium waste container in the hood.
5. Transfer the worms to an iced glass dissecting platform such as a small glass Petri dish. Cut worms with a scalpel blade (#15) in the pharynx and in the vulval region. We often cut only in the vulval area.
6. Transfer worms back to the vial.
7. Replace buffer with ice-cold fix B.
8. Incubate 3 hrs at 4°C. You can leave this overnight and it wont hurt. After this the worms are fairly brittle and usually much darker and somewhat translucent.
9. Embed[4] in 2% low melt agarose made in H_2O.
10. Allow agar to harden for an hour at 4ºC. Trim the agar to a block of ~2 mm (h) x 3 mm (l) x 3 mm (w) or smaller. Keep in mind the blocks must fit in the final mold that you will be using in the plastic embedding.
11. Place blocks in glass vials with caps (EMS[8] catalog #64252) or scintillation vials
12. Dehydrate and move to resin. This next set of incubations is all carried out while gently rocking on a flat bed rocker to keep the solutions well agitated. Once you are in ethanol, the blocks can be hard to see so do washes carefully. If you have a tissue processor you can use it instead of manual changing and agitation. (Plan on ~3.5 hrs for 12-A to 12-N)

    A. 1% uranyl acetate[6]- 45 min (filtered!). I have heard that making fresh works better but I have not found significant difference. But I don't use it older than 1 year. Collect waste uranyl acetate in container in the hood.
    B. dH_20[5]- 10 min.
    C. 50% EtOH-5 min (15 ml 100% EtOH+ 15ml dH_20[5])

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[a] This protocol is a modification of a protocol from Erik Jorgensen, University of Utah.

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D. 70% EtOH-5 min (21 ml 100% EtOH+ 9 ml dH_20)
E. 80% EtOH-5 min (24 ml 100% EtOH+ 6 ml dH_20)
F. 96% EtOH-5 min (28.8 ml 100% EtOH+ 1.2 ml dH_20)
G. 96% EtOH-5 min
H. 100% EtOH-10 min (Open fresh bottle 100% ethanol and add molecular sieves Fisher M564-500, type 3A, 8-12 mesh, effective pore size 3A. Beads can be reused by dehydrating them in the hood followed by optional gentle heating)
I. 100% EtOH-10 min
J. 100% EtOH-10 min
K. 1:1 PO:100% EtOH-10 min (propylene oxide: ethanol)
L. PO- 10 min
M. PO- 10 min
N. PO- 10 min
O. 2:1 PO:resin 4^+ hrs. Usually I make the 1/4 resin recipe.
P. 1:2 PO:resin 4^+ hrs.
Q. resin-6 hr. Can be done up to 14 hours but don't go longer otherwise it will be too viscous to remove. Usually I try to stick to under 8 hours.
R. resin- 8 hr. Use fresh resin and change old viscous one. Can be done longer. If Q is 14 hours then I just do two short resin washes for 4 hours each.

13. Embed and polymerize at 60ºC for 2-3 days. To embed make fresh resin again and use a matchstick to orient your block. Spray the mold with PTFE releasing agent (EMS[8] 50485-00), cut small papers in which you have the genotype typed or penciled in. We use EMS[8] beem flat molds (cat #70904).

Solutions

Buffer: 10mM HEPES (dilute from a 200 mM stock made from 12.5 g HEPES in 250 ml H_2O adjusted to pH 7.4). Filter.

Fix A: 0.67% gluteraldehyde/0.67% Osmium in 10mM HEPES (53.6 μl 25% Gluteraldehyde, 335 μl 4% osmium, 100 μl 200mM HEPES, 1.5 ml dH_2O)

Fix B: 2% osmium in 10mM HEPES (335 μl 4% osmium, 100 μl 200mM HEPES, 1.55 ml dH_2O)

Stain: 1% uranyl acetate[6] in dH_2O. Filter before use.

PBS (Phosphate Buffered Saline) 10 X stock solution: 80g, NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 800 ml dH_2O, adjust to pH 7.4 with HCl, fill to 1 liter and filter.

Resin: Epon 812 from Ted Pella[7] (number in parenthesis is what it comes to when you weigh it out one after the other. Mix until schlieren lines disappear. But, do not mix too much otherwise bubbles will form in your block.)

Full recipe	1/2 recipe	1/4 recipe
13 gm Eponate 812	6.5	3.25
5.5 gm NMA	2.75 (9.25)	1.375 (4.625)
7.5 gm DDSA	3.75 (13)	1.875 (6.5)
0.7 ml BDMA	0.35ml	0.175ml

use 0.8 ml BDMA if not hardening. An alternative accelerating agent is 0.5 ml DMP30

Chemical sources:

25% gluteraldehyde (EMS[8] cat #16220). Store after opening at 4°C sealed with parafilm. We use for up to 2 years if well sealed.

4% osmium[3] in vials from (EMS[8] cat #19150). Store after opening at 4°C by moving osmium to a 15 ml screw cap tube in a special jar used to store volatiles. Stop using after osmium turns black.

dH_20 Millipore water of 18 mOhm resistance, filtered.

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[1] Washes before agar embedding in this protocol are all done in Glass screw-cap vials. We use KIMAX® Micro-Vial, KG-33 Borosilicate Glass, Screw Thread, Graduated, with Open Top Closure and PTFE (Catalog no. 60700). After addition of the wash solution, the vial is capped, and then agitated to mix worms, then the worms are pelleted to the bottom of the vial by spinning at low speed in a clinical centrifuge for 5 to 10 second. The solution is then removed with a Pasteur pipette and place in an appropriate hazardous waste container.

[2] One common approach we take for timing of the protocol is to do step 1 to 8 day 1, and step 9 to start of 12-P on day 2, and finish embedding on day 3.

[3] Osmium is dangerous. It can coat your retina. Use osmium in the hood with goggles on. We put waste osmium and gluteraldehyde in hazardous waste containers in the hood.

[4] The idea is end up with 3 to 5 worms aligned next to each other in an agar block so that they are easy to manipulate during embedding and sectioning. In brief, worms are aligned on an agar pad in a small amount of buffer, the buffer is wicked away with a Kim Wipes, and the worms are gently covered with agar flowing from a dropper. This is the hardest step of the procedure and takes some practice. Make 2% low melting agarose in dH_20, melt agarose in microwave and stick stock bottle at 60-65° C. Keep a small working stock in a test tube in a 42° C temp block. I prepare a several large pads on a 15 cm Petri plate by placing large drops of agarose on the dish. The worms are easier to

move on the agarose than on a plastic surface. After the pads have hardened, transfer 3 to 5 worms onto one of the agar pads. We transfer worms using 50 μl capillary pipettes using a Clay-Adams pipettor or a mouth pipetting device, or a Pasteur pipette and bulb. Remove most of the buffer using the pipettor, then oriented the worms using a worm pick. Then remove much of the remaining liquid as possible from around the worms using a Kim Wipe, then gently allow melted agarose to flow over the worms from a Pasteur pipette tip or dropper. Be as gentle as possible when dropping the agarose onto the worms and have the agarose just above gelling temperature. Ideally 3 to 5 worms end up side by side virtually touching in agar. We typically make 3 to 5 such grouping of worms.

[5] All water should be filtered.

[6] Uranyl acetate is naturally radioactive. However, there is no need to use protection.

[7] Ted Pella, Inc. P.O. Box 492477, Redding, CA 96049-2477. Tel: 530-243-2200; 800-237-3526 www.tedpella.com

[8] Electron Microscope Sciences P.O. Box 550, 1560 Industry Road, Hatfield, PA 19440, Tel #: 215-412-8400, Fax #: 215-412-8450 www.emsdiasum.com

Last update 12-1-04