In situ hybridization on whole mount embryos of *C. elegans**

Tomoko Motohashi, Keiko Hirono, and Yuji Kohara§

Genome Biology Lab, Center for Genetic Resource Information, National Institute of Genetics, Mishima 411-8540, Japan

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*Edited by Ikuko Muramatsu, Masumi Obara, Wakako Shimizu, Aya Hamakawa, and Yuji Kohara. Last revised April 27, 2005. Published May 19, 2015. This chapter should be cited as: Motohashi T., Hirono K., and Kohara Y. *In situ* hybridization on whole mount embryos of *C. elegans* (May 19, 2015), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.176.1, http://www.wormbook.org.

§To whom correspondence should be addressed: Email: Ikuko Muramatsu, isugiura@lab.nig.ac.jp, Yuji Kohara, ykohara@lab.nig.ac.jp

1. Preparation of DIG-labeled DNA probes for *in situ* hybridization

1.1. DIG-labeling by linear PCR

1. Make the following reaction mix (total 10 µl):

distilled water	4.4 µl
anchored oligo dT primers*	3.0 µl
10 × Taq buffer	1 µl
Taq polymerase	0.1 µl
10 × DIG-dUTP/dNTP mix**	1.5 µl
cDNA insert (> 3.5 ng/µl) amplified from yk clone using T7/T3 primers	0.6 µl

* anchored oligo dT primers:

5.3 µM $(dT)_{17}$dG/5.3 µM $(dT)_{17}$dC/21.3 µM $(dT)_{17}$dA (This is to avoid the effect of poly-A stretch. Other vector primers may be used.)

** 10 × DIG-dUTP/dNTP mix:

0.35 mM DIG-dUTP/0.65 mM dTTP/1mM each d(A, G, C)TP

2. Subject to thermal cycling:

   hot start at 95 °C for 45 sec

   95 °C × 15 sec

   45 °C × 1 min

   72 °C × 1 min

   50 cycles

3. Add 10 µl of 10 mM EDTA

4. Pass through G-50 spin colum chromatography (ca. 250 µl bed volume)

5. Add 5 µl of TSE

1.2. Chopping the probes by DNaseI digestion

6. Make the following reaction mix (total 25 µl) on ice:

The G-50 elutate	20 µl
10 mg/ml Salmon sperm DNA	1 µl
distilled water	0.5 µl
10 × DNase buffer*	2.5 µl
DNaseI (16 µg/ml)**	1 µl

* 10 × DNase buffer: 0.5 M TrisHCl pH 7.5, 0.1 M $MgCl_2$

** Dilute stock solution (1 mg/ml) with 0.1 M NaCl.

(Note : Best size of probes is about 100 bases. Longer probes may cause high background. The concentrations of the enzyme should be optimized by pilot experiments.)

7. Incubate at 37 °C for 30 min
8. Transfer on ice
9. Add 5 µl of 0.1 M EDTA
10. Heat at 75 °C for 5 min
11. Check the size by alkaline agarose gel electrophoresis and DIG detection, if necessary
12. Store frozen

2. Fixation of embryos from a small number of worms

1. Take siliconized 1.5 ml eppendorf tubes
2. Place about 100 µl of distilled water on the (inside) top of the lids
3. Pick and transfer 40-50 gravid worms into the water. If you need very late stage embryos:
    a) Add 50 µl of suspension of *E. coli* OP50 in S-basal
    b) Cover the lid with the body of the tube
    c) Let stand at 20 °C overnight
4. Spin down the worms.
5. Add equal volume of 2 × alkaline-bleach solution and mix well
6. Leave at r.t. for 10 min to dissolve the adult bodies
7. Add 1 ml of M9 buffer at 4 °C
8. Centrifuge at 2500 rpm for 30 sec at 4 °C in a swing rotor
9. Remove the sup carefully, leaving about 100 µl of the sup to avoid removing the embryos
10. Repeat steps 7-9 three more times
11. Add an equal volume of 3 mg/ml chitinase
12. Mix and incubate at r.t. for 3 min
13. Spin at 2500 rpm for 30 sec at 4 °C
14. Reduce the volume to about 50 µl
15. Transfer the embryos to a poly-L-lysine coated 3-well slide using a siliconized pipette tip
16. Add a half volume of 4% gelatin, 2% BSA, and mix gently by pipeting

17. Let stand for several minutes to allow the embryos settled down to the bottom
18. Cover with a cover slip (24 × 40 mm)
19. Place it on the top of a dry ice block
20. Freeze for 7 min at -70 °C
21. Peel off the cover slip quickly
22. Soak the slide in methanol cooled at –20 °C for 5 min
23. Rehydrate by soaking the slide in the series of the following solutions pre-cooled at 4 °C:

methanol	for 5 min
methanol:formaldehyde-Hepes-PBS* = 35:15	for 2 min
methanol:formaldehyde-Hepes-PBS* = 25:25	for 2 min
methanol:formaldehyde-Hepes-PBS* = 15:35	for 2 min
formaldehyde-Hepes-PBS*	for 20 min

*formaldehyde-Hepes-PBS	
Hepes	200ml
10xPBS	25ml
formaldehyde	25ml

24. Dehydrate by soaking the slide in the series of the following solutions at r.t.:

ethanol:PBS=15:35	for 5min
ethanol:PBS=25:25	for 5min
ethanol:PBS=35:15	for 5 min
ethanol	for 5 min × 2 times

25. Store in ethanol at –20 °C.

3. Large scale fixation of embryos

3.1. Harvesting of embryos

1. Get a plenty of worms from a mixed stage population
2. Wash the worms 2 times with M9 buffer
3. Collect L1-L3 by sieving through 50 µm Nylon mesh
4. Allow the collected worms to grow to young adults in liquid culture
5. Take 1 ml packed worms from the culture, which will give 8-15 slides for *in situ*
6. Resuspend the worms in 4 ml water in a 15 ml Falcon tube (clear type)

7. Add 5 ml of 2 × alkali-bleach solution, mix well and let stand for 10 min
8. Force the worms out through a 23-gauge needle onto nylon mesh
9. Collect embryos by spinning the filtrate at 800 × g using a swing rotor
10. Wash the embryos 4 times with M9 and transferred into a siliconized eppendorf tube

3.2. Fixation

1. Take 100 µl (packed volume) of the embryos and adjust the volume to 200 µl with M9
2. Add 200 µl of yatalase (15 mg/ml in 0.3 M mannitol) and vigourously shake for 75 sec
3. Wash the embryos 3 times with EH buffer (Embryo Handling buffer)
4. Wash the embryos with Basal EH buffer
5. Resuspend the embryos in 1 ml of Basal EH buffer. (Note: For success, it is desired that 20-30% of embryos are devitellinized at this step)
6. Place 30 µl/well of Basal EH buffer onto each well of poly-L-lysine coated 8-well slides
7. Dispense 5 µl/well of the embryo suspension into the buffer at each well
8. Let stand for 10 min at 4 °C to settle the embryos to the bottom
9. Remove the buffer, and immediately immerse in methanol at –20 °C for 5 min
10. Rehydrate the embryos by immersing the slides in the follwoing series at 4 °C. The solutions must be pre-cooled at 4 °C.

methanol	5 min
methanol:3.7% formaldehyde in hepes-PBS = 7:3	2 min
methanol:3.7% formaldehyde in hepes-PBS = 1:1	2 min
methanol:3.7% formaldehyde in hepes-PBS = 3:7	2 min
3.7% formaldehyde in hepes-PBS	75 min at 22 °C

11. Dehydrate the embryos by immersing the slides in the following series at r.t.

ethanol:PBS = 3:7	5 min
ethanol:PBS = 1:1	5 min
ethanol:PBS = 7:3	5 min
ethanol	5 min × 2 times

12. Store in ethanol at –20 °C.

4. Hybridization and signal detection

4.1. Proteinase K treatment

1. Rehydrate the embryos by immersing the follwoing ethanol series:

0.03% H2O2 in ethanol:PBS = 7:3	2 min
ethanol:PBS = 1:1	5 min
ethanol:PBS = 3:7	5 min

2. Wash the slides once by immersing in PBT for 5 min. *For late stage embryos, additional HCl treatment is effective, which can cut glycosid bonds of the proteoglycan that appear on late stage embryos.
    a) Immerse the slides in 0.2 N HCl for 20 min at r.t.
    b) Wash the slides 2 times in PBT for 5 min
3. Immerse the slides in proteinase K (10 µg/ml in PBT) and incubate at r.t. for 11 min
4. Stop the digestion by immersing the slides in 2 mg/ml glycine in PBT for 2 min
5. Wash the slides twice by immersing them in PBT for 2 min each
6. Refix by immersing the slides in 3.7% formaldehyde in hepes-PBS at r.t. for 50 min
7. Wash the slides twice in PBT for 5 min each
8. Immerse the slides in 2 mg/ml glycine in PBT at r.t. for 5 min
9. Wash the slides in PBT for 5 min

4.2. Pre-Hybridization

1. Immerse the slides in the following series of mixtures;

50% formamide, 5 × SSC, heparin, 0.1% Tween:PBT = 1:1	10 min
50% formamide, 5 × SSC, heparin, 0.1% Tween	10 min

2. Wipe off the slides
3. Draw a rectangle surrounding the sample wells using a IMMUNO pen to make a ridge
4. Add 250 µl of heat denatured (at 99 °C for 10 min and quickly chilled for 5 min) hybridization solution for each 8-well slide
5. Place the slide in a moist chamber containing a paper towel wetted with 50% formamide, 5 × SSC. (No need to use coverslips)
6. Incubate at 48 °C for 1 hr

4.3. Hybridization

1. Add 50 µl of heat-denatured DNA probes for each slide. (The final concentrations of probes is about 0.06 µg/ml)
2. Cover the slide with a parafilm coverslip to reduce evaporation
3. Incubate the slides at 48 °C overnight in the moist chamber

4.4. Washing

1. Wash the slides in the following series of washing solutins at 48 °C with slight agitation

50% formamide, 5 × SSC, heparin, 0.1% Tween:PBT = 1:1 (First washing is performed in separate containers for every 2 min slides)	
50% formamide, 5 × SSC, heparin, 0.1% Tween:PBT = 1:1	10 min × 2 times
0.8 × PBS, 0.1% CHAPS	20 min × 4 times

2. Wash the slides twice in PBT for 5 min at r.t. to remove CHAPS.

4.5. Probe detection

1. Incubate the slides in PBtr (PBS, 0.1% Triton-X100, 0.1% BSA, 0.01% NaN_3) for 1.5 hr at r.t.
2. Cover the embryos with 250 µl of anti-DIG conjugate (dilute 1:2500)/8-well slide
3. Incubate for 2 hrs at r.t. in a moist chamber. (no need to use coverslips)
4. Wash the slides with PBtr 4 times with slight agitation
5. Wash the slides with the staining buffer (see reagents) twice for 5 min each at r.t.
6. Color development
    a) Mix 180 µl of NBT and 140 µl of BCIP in 40 ml of staining buffer.
    b) Immerse the slides in the mixture for 1 hr at 22 °C in the dark, monitoring the extent of the staining.
7. Wash the slides three times with PBS, 20 mM EDTA to stop the reaction.
8. If necessary, incubate the slides in 1 µg/ml DAPI in Tris buffer at 4 °C for 30 min.

4.6. Mounting

4.6.1. Permanent mount 1

1. Add about 90 µl of “MOUNT-QUICK AQUEOUS” onto the embryos on the slide
2. Cover with a coverslip
3. Let stand one day to dry up
4. Seal up the edge of the coverslip using nail varnish

4.6.2. Permanent mount 2

1. Dehydrate with the following ethanol series:

ethanol:PBS = 3:7	5 min
ethanol:PBS = 1:1	5 min
ethanol:PBS = 7:3	5 min
ethanol	5 min × 2 times

2. Wash once with ethanol:Histo-Clear (National Diagnostics) = 1:1
3. Wash once with Histo-Clear
4. Add drops of Mount-Quick onto the embryos and cover with a coverslip
5. Leave the slide at 40 °C for several hours

(Note : Hybridization signals by this method tend to be weaker than those by other methods and to diffuse, but preservation of morphology is better than other methods)

4.6.3. Glycerol mount

1. Add drops of 90% glycerol, 10 mM Tris, 1% n-propylgallate onto the embryos
2. Cover with a coverslip which are dotted with vaselin:solid paraffin = 9:1 at the 4 corners as spacer

5. Reagents

M9

KH_2PO_4	3 g
Na_2HPO_4	6 g
1M $MgSO_4$	1 ml
Add DW to total 1 liter and autoclave	

S-basal

NaCl	69 g
1M K-PO_4 (pH6)	100 ml
cholesterol (5 mg/ml in EtOH)	2 ml
Add DW to total 2 liter and autoclave	

2 × alkali-bleach solution

NaClO	3.0 ml
5M KOH	2.5 ml
DW	19.5 ml

PBS

NaCl	13 7 mM
KCl	2.7 mM
Na_2HPO_4	4.3 mM
KH_2PO_4	1.5 mM
Adjust pH to 7.2 and autoclave	

PBT

PBS + 0.1% Tween 20

EH buffer (Embryo Handling buffer)

mannitol	0.3 M
Hepes pH 7.2	50 mM
NaCl	10 mM
$MgCl_2$	10 mM
EGTA	0.04%
NH_4NO_3	2 mM
gelatin	0.1%
DTT	2 mM

Basal EH buffer

(= EH buffer without EGTA, NH_4NO_3, gelatin and DTT)

Glycine in PBT

Glycine	2 mg/ml in PBS
	autoclave, then add 0.1% Tween 20

3.7% Formaldehyde in hepes-PBS

hepes buffer*:formalin:10 × PBS = 8:1:1

***hepes buffer**

Hepes	100 mM
$MgSO_4$	2 mM
EGTA	0.04%
Add NaOH to pH6.9 and autoclave	

Hybridization solution

deionized formamide	50%
SSC (pH7, autoclaved)	5×
sonicated salmon testis DNA	100 µg/ml
yeast tRNA	100 µg/ml
heparin	100 µg/ml
Tween 20	0.1%

yatalase (15 mg/ml) and **chitinase** (1 mg protein/ml = 5 mg crude/ml)

1. Dissolve powder of yatalase (TAKARA No.T017) or chitinase (SIGMA No. C-6137) in 0.3 M mannitol, 50 mM Hepes pH 7.2, 10 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT
2. Filtrate through a 0.45 µm syringe filter.
3. store at –20 °C.

Digoxigenin-11-dUTP (Roche 1570013)

PBT

0.1% Tween-20 in PBS (0.01% DEPC treated)

PBtr

0.1% BSA (Fraction V), 0.1% Triton X-100 in PBS proteinase K stock solution

20 mg Proteinase K (Roche 30U/mg)/ml water

Staining buffer (Alkaline phosphatase reaction buffer)

NaCl	100 mM
$MgCl_2$	5 mM
TrisHCl pH 9.5	100 mM
Levamisol	1 mM
Tween-20	0.1%

poly-L-lysine coated slides

1. Immerse glass slides in solution of kitchen detergent for 20 min
2. Wash in tap water for 1 hr
3. Wash in ion-exchanged water
4. Autoclave and dry at 80 °C
5. Drop poly-L-lysine solution (SIGMA P-8920) onto individual wells of the slides
6. Leave for 25 min

7. Aspirate off the excess solution (only when 3-well slides are used)
8. Dry at 65 °C for 1 hr

Parafilm coverslips

1. Dribble beads of rubber cement along the edge of square pieces of parafilm
2. Dry briefly at 35-40 °C