Automated whole-mount in situ hybridization on developmental stages of Ciona intestinalis for the identification of recessive mutations with subtle phenotype

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The systematic identification of spontaneous mutations with subtle phenotypes in developmental stages of *Ciona intestinalis* (Ascidiacea, Tunicata) is facilitated by the simultaneous utilization of multiple cell type markers. To this aim, we developed a high-throughput protocol for triple whole mount *in situ* hybridization by automatization with the Intavis InsituPro system. This approach can be performed in 30-well plates and with similar conditions for all antisense riboprobes, including hybridization temperature and comparable mRNA staining intensity.

Note: all steps at RT and using DEPC water except where indicated.

1. Rehydrate larvae 2 x 10 min each in 250 µl of 50% and 30% EtOH.
2. Wash 3 x 7 min each in 250 µl of 1X PBT.
3. Postfixation 1 hr in 250 µl of 4% PFA in 1X PBS.
4. Wash samples 3 x 7 min each in 250 µl of 1X PBT.
5. Incubate 30 min in 250 µl of 1X PBT containing 4 µg/ml Proteinase K at 37°C water bath.
6. Refix 1 hr in 250 µl of 4% PFA in 1X PBS.
7. Wash 3 x 7 min each in 250 µl of 1X PBT.
8. Wash 3 x 10 min in 250 µl of 0.25% acetic anhydride, 0.1 M triethanolamine.
9. Wash 3 x 7 min each in 250µl of 1X PBT.
10. Incubate 20 min in 250 µl of 1:1 hybridization solution and 1X PBT.
11. Incubate for 30 min in 250 µl of hybridization solution.
12. Incubate for 2 hr in 250 µl of hybridization solution at 55°C.
13. Incubate around 18 hrs in 250µl of hybridization solution containing 0.3-0.6 ng/ml DIG-labelled riboprobes (e.g. *glyr*, *arrestin*, *six3/6*) at 55°C (Notes: DIG-labelled riboprobes concentration to be estimated by dot blot analysis).
14. Wash at 55°C in:
    2 x 15 min in 250 µl of washing buffer 1;
    2 x 15 min in 250 µl of washing buffer 2.
15. Wash 3 x 10 min in 250 µl of Solution A at 37°C.
16. Incubate 30 min at 37°C in 250 µl of Solution A containing 20 µg/ml RNAseA.
17. Wash 15 min at 37°C in 250 µl of Solution A.

Reagents: *EtOH, DEPC and sterile H2O, TWEEN-20, 10x PBS in DEPC H2O, Proteinase K, paraformaldehyde, acetic anhydride, triethanolamine, formamide, SSC, tRNA, Denhard's, heparin, NaCl, Tris pH 8.0, EDTA, RNAseA, blocking reagent, sheep serum, alkaline phosphatase, anti-Dig coniugated antibody, Tris pH 9.5, MgCl2, NBT, BCIP.*

Equipment: *Intavis InsituPro system, 24 - well plates, Pasteur pipettes, 50 ml Falcon tubes, Parafilm, 2 ml vials.*

ASSEMBLE
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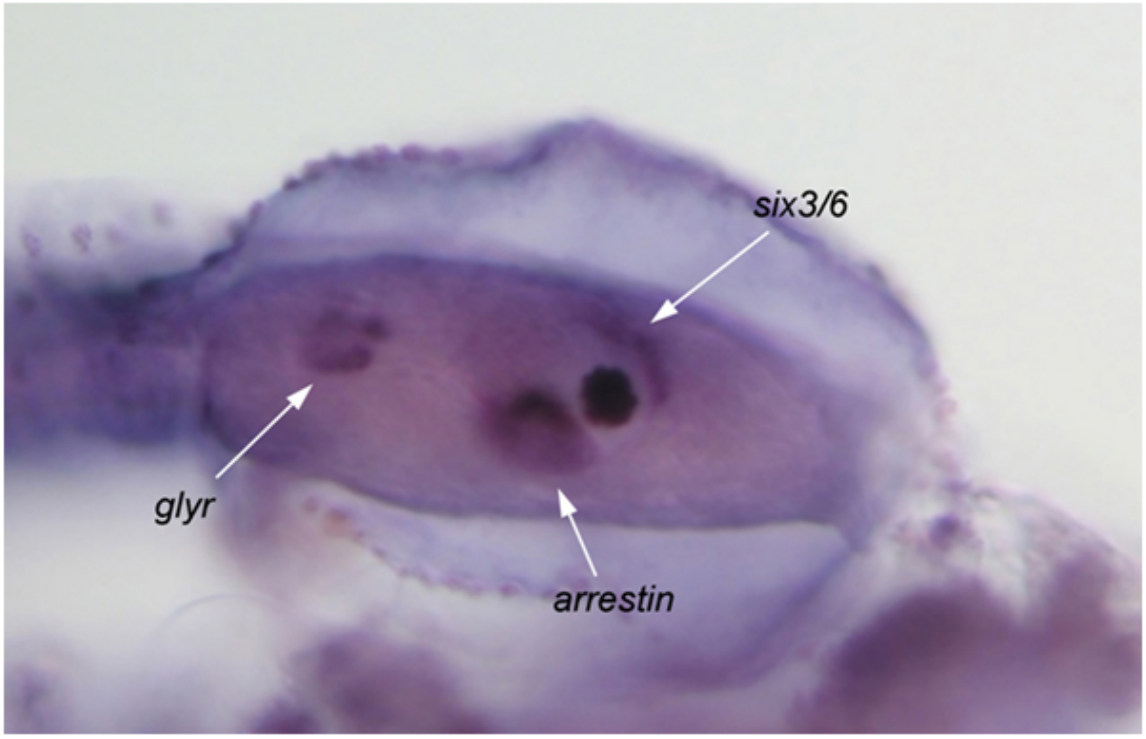
18. Wash at 55°C in:
    1 x 20 min in 250 µl of washing buffer 2;
    2 x 15 min in 250 µl of washing buffer 3.
19. Wash 15 min in 250 µl of 1:1 1X SSC in PBT.
20. Wash 4 x 7 min in 250 µl of 1X PBT in sterile H2O.
21. Incubate 1 hr in 250 µl of blocking buffer.
22. Incubate 5 hrs in 250 µl of fresh blocking buffer containing 1:2000 Alcaline Phosphatase anti-DIG-antibody.
23. Wash 11 x 20 min in 250 µl of 1X PBT in sterile H_2O.
24. 2 x 10 min in 250 µl of AP buffer.

    FOLLOWING STEPS TO BE DONE MANUALLY:

25. Incubate in 1 ml AP buffer containing 4.5 µl of NBT and 3.5 µl of BCIP.
26. Stop staining reaction with 1X PBT in sterile H_2O.



SOLUTIONS (all prepared fresh except where indicated)

50% ETOH IN DEPC H_2O

Reagent	Quantity (for 50 ml)	Final concentration
100% EtOH	25 ml	50%
DEPC H_2O	up to 50 ml	

Mix gently and store at room temperature.

30% ETOH IN DEPC H_2O

Reagent	Quantity (for 50 ml)	Final concentration
100% EtOH	15 ml	30%
DEPC H_2O	up to 50 ml	

Mix gently and store at room temperature.

1X PBT IN DEPC H_2O (or sterile H_2O)

Reagent	Quantity (for 50 ml)	Final concentration
PBS	5 ml of 10X PBS stock	1X
100% TWEEN-20	50 µl	0.1%
DEPC H_2O (or sterile H_2O)	up to 50 ml	

Mix gently and store at room temperature.

4% PFA IN 1X PBS

Reagent	Quantity (for 50 ml)	Final concentration
Paraformaldehyde	2 grams of paraformaldehyde	
PBS	5 ml of 10X PBS stock	1X
DEPC H_2O	up to 50 ml	

Seal with Parafilm and shake the 50 ml Falcon tube.
Incubate at 65°C in a water bath for 45 min (shake each 10 min until paraformaldehyde is dissolved).
Filter solution at 0.22 µM.
Aliquot in 2 ml vials.
Store aliquots at -20°C.

1X PBT IN DEPC H_2O CONTAINING 4µg/ml PROTEINASE K

Reagent	Quantity (for 50 ml)	Final concentration
PBS	5 ml of 10X PBS stock	1X
100% TWEEN-20	50 µl	0.1%
Proteinase K	20 µl of 10 mg/ml Proteinase K	4 µg/ml
DEPC H_2O	up to 50 ml	

Mix gently.

0.25% ACETIC ANHYDRIDE, 0.1M TRIETHANOLAMINE

Reagent	Quantity (for 50 ml)	Final concentration
100% acetic anhydride	125 µl	0.25%
triethanolamine	5 ml of 1 M triethanolamine stock pH 8.0	0.1 M
DEPC H_2O	up to 50 ml	

Mix gently.

HYBRIDIZATION SOLUTION

Reagent	Quantity (for 50 ml)	Final concentration
100% formamide	25 ml	50%
SSC	12.5 ml of 20X SSC stock	5X
tRNA	250 µl of 10 mg/ml tRNA	50 µg/ml
Denhardt's	5 ml of 50X Denhardt's stock	5X
100% TWEEN-20	50 µl	0.1%
heparin	50 µl of 50 mg/ml heparin stock	50 µg/ml
DEPC H_2O	up to 50 ml	
Mix gently and store at -20°C.		

WASHING BUFFER 1

Reagent	Quantity (for 50 ml)	Final concentration
100% formamide	25 ml	50%
SSC	10 ml of 20X SSC stock	4X
100% TWEEN-20	50 µl	0.1%
DEPC H_2O	up to 50 ml	
Mix gently.		

WASHING BUFFER 2

Reagent	Quantity (for 50 ml)	Final concentration
100% formamide	25 ml	50%
SSC	5 ml of 20X SSC stock	2X
100% TWEEN-20	50 µl	0.1%
DEPC H_2O	up to 50 ml	
Mix gently.		

SOLUTION A

Reagent	Quantity (for 50 ml)	Final concentration
NaCl	5 ml of 5 M NaCl stock	0.5 M
Tris pH 8.0	500 µl of 1 M Tris pH 8.0 stock	10 mM
EDTA	500 µl of 0.5 M EDTA stock	5 mM
100% TWEEN-20	50 µl	0.1%
DEPC H_2O	up to 50 ml	
Mix gently.		

SOLUTION A CONTAINING 20µg/ml RNAseA

Reagent	Quantity (for 50 ml)	Final concentration
NaCl	5 ml of 5 M NaCl stock	0.5 M
Tris pH 8.0	500 µl of 1 M Tris pH 8.0 stock	10 mM
EDTA	500 µl of 0.5 M EDTA stock	5 mM
100% TWEEN-20	50 µl	0.1%
RNAseA	100 µl of 10 mg/ml RNAseA stock	20 µg/ml
DEPC H_2O	up to 50 ml	
Mix gently.		

WASHING BUFFER 3

Reagent	Quantity (for 50 ml)	Final concentration
100% formamide	25 ml	50%
SSC	1.25 ml of 20X SSC stock	0.5X
100% TWEEN-20	50 µl	0.1%

DEPC H_2O	up to 50 ml

Mix gently.

1X SSC IN PBT

Reagent	Quantity (for 50 ml)	Final concentration
PBS	5 ml of 10X PBS stock	1X
SSC	2.5 ml of 20X SSC stock	1X
100% TWEEN-20	50 µl	0.1%
DEPC H_2O	up to 50 ml	

Mix gently.

BLOCKING BUFFER

Reagent	Quantity (for 50 ml)	Final concentration
Blocking reagent	2.5 ml of 10% blocking reagent stock	0.5%
100% Sheep Serum	2.5 ml	5%
PBS	5 ml of 10X PBS stock	1X
100% TWEEN-20	50 µl	0.1%
DEPC H_2O	up to 50 ml	

Mix gently.

BLOCKING BUFFER CONTAINING 1:2000 ALCALINE PHOSPHATASE ANTI-DIG-ANTIBODY

Reagent	Quantity (for 50 ml)	Final concentration
Blocking reagent	2.5 ml of 10% blocking reagent stock	0.5%
Alcaline Phosphatase anti-DIG-antibody	25 µl of 0.75 U/µl AP anti-DIG-antibody stock	0.375 mU/µl
100% Sheep Serum	2.5 ml	5%
PBS	5 ml of 10X PBS stock	1X
100% TWEEN-20	50 µl	0.1%
DEPC H_2O	up to 50 ml	

Mix gently.

AP BUFFER

Reagent	Quantity (for 50 ml)	Final concentration
NaCl	1 ml of 5 M NaCl stock	100 mM
Tris pH 9.5	5 ml of 1 M Tris pH 9.5 stock	100 mM
$MgCl_2$	2.5 ml of 1 M $MgCl_2$ stock	50 mM
100% TWEEN-20	50 µl	0.1%
Sterile H_2O	up to 50 ml	

Mix gently.